Yeast contamination of meats and processing equipment

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YEAST CONTAMINATION OF MEATS AND PROCESSING EQUIPMENT

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SUMMARY

An extensive review of a diffuse literature on yeast spoilage of foods is presented in Chapter 1. Stress-tolerant yeasts commonly spoil foods which impose extremes of environments such as high acidity, low a_w etc. A further group of yeasts, termed 'opportunist spoilers', are common contaminants of a wide range of foods. The factors underlying the change in status from food-borne to food spoilage have not been defined in detail, but a reduction in bacterial contamination seems to be involved. Of the 114 yeast species isolated in many studies of meats, most were members of the following six genera; Candida, Debaryomyces, Cryptococcus, Rhodotorula, Trichosporon and Pichia. Violet Red Bile Glucose Agar supplemented with oxytetracycline was found to be a good screening medium because it differentiated between the six 'meat genera' on the basis of colony colour and texture. A simplified classical key specifically for yeasts in meats was devised. Only 12 tests were necessary and 3 weeks required for complete identification.

It was established that the heat treatment of skinless sausage, a product commonly spoilt by yeasts, had a pasteurising effect on surface contamination. During storage yeasts rapidly outgrew bacteria, particularly during the warmer months. At this time there was a succession in yeast colonisation such that, when spoilage was manifested by a yellow slime, Candida zeylanoides, Debaryomyces hansenii and Candida lipolytica were the dominant organisms.

An investigation of poultry processing plants revealed a linear distribution of yeast types on equipment along the processing line. Filamentous yeasts, Trichosporon cutaneum in particular, were the dominant ones at slaughter and plucking. At evisceration the flora changed. Yeasts with smooth colonies appeared and persisted during chilling, portioning and packaging. Whole and portioned chicken were contaminated with the same yeast types as those which occurred in the latter stages of processing, namely; Candida japonica, Cryptococcus laurentii, Candida lipolytica, Rhodotorula glutinis. Trichosporon cutaneum and Sporobolomyces salmonicolor. Post-processing irradiation (2.5 kGy) of fresh portions resulted in a marked kill-off of bacteria. Yeasts were more resistant. Long-term deep frozen storage exerted a highly significant ($P < 0.001$) lethal effect on bacterial contaminants on portions. There was no significant change in the levels of yeast contamination during 3 months frozen storage (-18°C). At this time the yeast flora was dominated by Cryptococcus laurentii.

ABBREVIATIONS

The following abbreviations are used for names of genera:

Aciculoconidium	*Ac.*	*Pachysolen*	*Pa.*
Ambrosiozyma	*A.*	*Pachytichospora*	*P'spora*
Arthroascus	*Ar.*	*Phaffia*	*Ph.*
Brettanomyces	*Br.*	*Pichia*	*P.*
Bullera	*B.*	*Pityrosporum*	*Pit.*
Candida	*C.*	*Rhodosporidium*	*Rhodosp.*
Citeromyces	*Cit.*	*Rhodotorula*	*Rh.*
Clavispora	*Cl.*	*Saccharomyces*	*Sacch.*
Cryptococcus	*Cr.*	*Saccharomycodes*	*S'codes*
Cyniclomyces	*Cyn.*	*Saccharomycopsis*	*S.*
Debaryomyces	*Deb.*	*Sarcinosporon*	*Sar.*
Dekkera	*D.*	*Schizoblastosporion*	*Schizobl.*
Fibulobasidium	*Fib.*	*Schizosaccharomyces*	*Schiz.*
Filobasidiella	*Fib.*	*Schwanniomyces*	*Schw.*
Filobasidium	*Fil.*	*Sirobasidium*	*Sir.*
Guilliermondella	*G.*	*Sporidiobolus*	*Sporid.*
Hanseniaspora	*H'spora*	*Sporobolomyces*	*Sp.*
Hansenula	*H.*	*Sporopachydermia*	*Sporop.*
Holtermannia	*Holt.*	*Stephanoascus*	*Steph.*
Issatchenkia	*I.*	*Sterigmatomyces*	*St.*
Kloeckera	*Kl.*	*Sympodiomyces*	*Symp.*
Kluyveromyces	*K.*	*Torulaspora*	*T'spora*
Leucosporidium	*Leu.*	*Torulopsis*	*T.*
Lipomyces	*L.*	*Tremella*	*Trem.*
Lodderomyces	*Lod.*	*Trichosporon*	*Tr.*
Malassezia	*Mal.*	*Trigonopsis*	*Trig.*
Metschnikowia	*M.*	*Wickerhamia*	*W.*
Nadsonia	*N.*	*Wickerhamiella*	*Wick.*
Nematospora	*Nem.*	*Wingea*	*Wi.*
Ooosporidium	*O.*	*Zygosaccharomyces*	*Zygosacch.*

INTRODUCTION

An extensive literature review of yeasts as food spoilage organisms is presented in Chapter 1. These organisms commonly cause problems with highly acidic foods and/or those with a low water activity such as preserves and confectionery. Yeasts are common albeit minor contaminants of a wide range of foods which do not impose extreme environments, for example, yoghurts and meats. Although bacteria are the major contaminants of such foods, yeasts compete effectively with them and on occasions cause spoilage. The ratio of yeast to bacteria appears to be important in this context; if this is $\geq$ 1 : 1, then yeasts may well become dominant.

Yeasts in meats are mainly restricted to the following genera; Candida, Debaryomyces, Cryptococcus, Rhodotorula, Trichosporon and Pichia (Déak and Beauchat, 1987). Whilst previous work has concentrated on bacterial associations in meats, little attention has been paid to yeasts. The reasons for this are two-fold; firstly, bacteria are the dominant contaminants and the usual cause of spoilage, and secondly, because yeast numbers are generally low they are considered to be minor contaminants (Walker and Ayres, 1970). It is important to note that even though counts are low, the contribution in terms of biomass, of yeasts to the microbial association may be considerable (Dalton, 1984).

Yeasts are notoriously difficult to identify owing to the procedures which have evolved over the years. Chapter 2 considers appropriate schemes of classical (Kreger-van Rij, 1984) and

ecological (Davenport, 1987) yeast identification methods. In the present study, a differential medium was developed for screening yeasts and identification was accomplished using a simplified classical key devised specifically for yeasts in meats.

Chapters 3 and 4 present the results of studies of two systems involving yeasts, namely (1) the succession of surface yeast contamination of skinless sausage, a sulphited derivative of the British fresh sausage and (2), the linear distribution of yeast types on equipment along a poultry processing line. The skinless sausage is unique in that it is a sulphited meat which undergoes a heat (pasteurising) treatment during processing but without aseptic treatment post-processing. Despite the fact that it frequently spoils in the warmer months as a result of yeast growth, no systematic investigation of this has been recorded. In Chapter 3, yeast contamination of skinless sausage during processing and subsequent chill storage is described.

Investigations of yeast flora along sheep (Baxter and Illston, 1976) and pig (Dalton, 1984) processing lines revealed a distribution of organisms originating from the lairage and hides of animals. These organisms occurred sporadically on equipment and were often found on the processed meat. Production of poultry is of particular interest at the present time owing to the widespread concern about product contamination with pathogenic organisms. Changes in processing methods and post-processing treatments are being suggested in an attempt to reduce levels of Salmonella, yet little mention is made of the likely effect on the yeast flora. One proposal involves irradiating carcasses with a pasteurising dose of

2.5 kGy. Recent studies indicate that such a dose causes an increase in the proportion of yeasts in the overall contamination (Johannsen et al., 1984; Hughes and Patterson, 1988). Indeed, after such treatment, subsequent storage may result in yeast spoilage. It is also noteworthy that frozen storage might elect for yeast survival/growth. Lowry and Gill (1984) found that although bacteria did not grow on lamb stored at -5°C for 10 weeks, yeasts did. In particular, Cryptococcus laurentii grew comparatively rapidly and accounted for over 90% of the yeast flora at this time. In Chapter 4 the types of yeast on equipment during poultry processing are identified. The flora on whole and portioned chickens was examined initially and after chill and frozen storage. The effect on levels of yeast and bacterial contamination of chicken portions was investigated, (1) after long-term deep frozen storage and (2), after post-processing irradiation.

CHAPTER 1

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E.A. Tudor and R.G. Board
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FOOD SPOILAGE YEASTS

This chapter presents an extensive literature review. It considers a range of foods in which yeasts commonly cause spoilage. Traditional foods liable to yeast spoilage impose environmental extremes, such as high acidity and low a_w, which are elective for yeasts. The various manifestations of spoilage are considered in terms of yeast biomass and physiology. Yeast problems associated with novel foods either based on traditional recipes, or arising from new processing methods are discussed. Yeasts which are common contaminants and occasionally cause spoilage of such foods are considered as opportunist spoilers. Factors contributing to their success are discussed.

I INTRODUCTION

The study of yeasts in the broad context of food microbiology lacks a conceptual framework. It is not generally appreciated, for example, that the storage of a commodity (eg. grain) forms a man-made ecosystem which as a consequence of frequent interference is immature, unstable and colonized by species (mainly micro-fungi) of limited specialisation, but capable of high growth and reproductive rates (Lacey, 1988a,b). Several examples of such man-made ecosystems will be discussed in this Chapter. In the preparation of a food product resources are made available for exploitation by micro-organisms often as a result of the destruction of biological structure. The organisms may have survived a manufacturing process or gained access to a "commercially sterile" product through careless post-processing practices. In ecological terms one is dealing with a destructive disturbance of ecosystems (Pugh and Boddy, 1988). If a food or a storage environment does not impose stress conditions, then selection favours microorganisms capable of rapid uptake of easily assimilable nutrients (rapid primary resource recapture). In the vast majority of cases the microbial associations at the time of spoilage will be dominated numerically by bacteria (Mossel and Ingram, 1955). In many instances, however, the ultimate biomass achieved by yeasts is not appreciably different from that of bacteria. Even so there is little evidence of yeasts contributing directly to spoilage of foods which do not impose stress conditions. Mossel and Ingram (1955) considered the possibility of bacterial growth in a food being aided by vitamin production by yeasts. As yet, however, there

are no outstanding examples of such synergism operating in food spoilage. In the context of food microbiology, it has been established that a wide range of food products harbour yeasts which do not contribute to spoilage (eg. Banks and Board, 1987; Deák and Beuchat, 1987). Examples of the occurrence of food-borne yeasts will be discussed in this Chapter but only to illustrate particular features such as the contribution of equipment or ingredients, for example, to product contamination or to highlight poorly defined technological innovations which may favour their growth.

Stress tolerant micro-organisms are selected when stress conditions obtain during either the processing or storage of a commodity or from the systems used to preserve a food. In general stress conditions favour the growth of yeasts and micro-fungi more than that of bacteria. This Chapter will consider yeasts which are exceptionally tolerant to several environmental extremes, with an emphasis being given to manufacturing practices contributing to their selection. The physiological adaptions which permit such organisms to cope with extreme conditions will not be considered.

A Factors allowing yeast growth

Studies of traditionally produced foods have identified four stress factors, often working synergistically, which favour yeast growth: low water activity (Beuchat, 1983); low pH (Ingram, 1958); low temperatures (Davenport, 1980a), and the presence, normally as the consequence of deliberate addition, of antibacterial agents (Warth, 1985, 1988). Of course the curtailment of bacterial

competition by these agents will also be conducive to yeast growth (Beuchat, 1983). As these factors also favour the growth of micro-fungi, another factor needs to be identified in order to account for the success of yeasts as spoilage organisms. According to Pitt and Hocking (1985) the physical state of an ingredient or food is important. On solid materials yeasts remain localised and their presence is generally masked by micro-fungi ramifying over the surface and, in many cases, penetrating the substrate. In liquids budding or less commonly fission, leads to the rapid dispersal of unicellular yeasts. Anoxia may also favour the growth of fermentative yeasts in preference to that of micro-fungi, especially when fermentable carbohydrates and vitamins are present. Of course spoilage will only occur if there is a depot of yeasts adapted to one or other of the environmental extremes noted above. Consequently studies of yeast spoilage of foods must take account of dispersal systems, a topic discussed when appropriate in this Chapter.

Consumer demand has led to changes in traditional products. In some instances changes have imposed novel selective conditions and resulted in yeast spoilage, dessert yoghurts providing an example. Yoghurts made in the traditional manner were microbiologically stable, at least in the short term, because during fermentation the lactic acid bacteria exhausted fermentable substrates, coagulated casein through acid production and removed dissolved oxygen. The increased demand for fruit-flavoured yoghurts has changed this situation. The addition of fruit and syrups to a traditionally fermented product introduces fermentable carbohydrates and

contaminants when Good Manufacturing Practices (GMP) are not observed. The contaminants are introduced at a time when the lactic acid bacteria have begun to decline in number and are less able to compete. The acid conditions resulting from the initial fermentation of lactose will of course favour the growth of yeasts. This topic is discussed more fully on pp. 74.

B Innovations in food-processing

The food industries in developed countries are currently in an innovative phase and four features impinging upon yeasts in foods need to be considered. One is the rapid adoption of Hazard Associated Critical Control Point Analysis (HACCP) in place of the earlier (and now largely discredited) Quality (Control) Assurance (QA) systems. QA is based on end product analysis for protecting a product against spoilage or a consumer against food-mediated disease. HACCP in contrast, highlights the importance of GMP both for raw ingredients even before entering the factory as well as during processing. It can be anticipated that increased attention will be given to food-borne yeasts through the application of HACCP even if at the outset the yeast counts are used mainly as indicators of ingredient quality, equipment hygiene and process control. In some circumstances a yeast to bacterial count of about 1:1 could well alert a producer to potential problems due to yeast spoilage. An example of this situation is discussed on pp. 96. The second feature of this innovative phase is the attempt to develop products having a long shelf-life at chill or, more especially, ambient storage. Some microbiologists are of the opinion that novel

preservative systems may hinder the growth of bacteria but not that of the slower growing yeasts or microfungi. Should their fears be realised, then yeasts now categorised as food-borne may well have to be considered as spoilage organisms. The third aspect is linked with technological innovations based on an improved understanding of a process. An example will be discussed in the section, Fruit and Vegetables (pp·57), where yeasts, formerly regarded as spoilage organisms, may well be used in the future to produce stable low-acid pickled vegetables. The fourth is associated with the possible use of irradiation to eliminate food poisoning bacteria. Thus irradiation of minced meat kills the majority of Gram-negative bacteria thereby diminishing bacterial competition to yeast growth (Johannsen et al., 1984-see also pp 96).

C Food microbiology and yeast taxonomy

Should the emergence of yeasts as important spoilage organisms occur as has happened with dessert-dairy products, then some sectors of the food industry will face problems. The average food microbiologist knows little about yeasts and this will be of immediate concern. Pitt and Hocking (1985) noted that over 120 species of yeasts from 30 genera - 50% of the recognised ones - have been associated with foods. As discussed elsewhere in this series on The Yeasts (Kreger-van Rij, 1987; Kurtzmann and Phaff, 1987) characterisation of yeasts, involving detailed studies of cell morphology and physiology, is time consuming. The identification of a yeast with existing classifications (Kreger-van Rij, 1987) is not without its pitfalls particularly as many organisms are known by

two names (see Appendix B). Deák and Beuchat (1987) have attempted to resolve some of the difficulties by devising a simplified identification method (10-15 tests) for use in food microbiology. In practice their system was based on a study of the 215 yeast species (cf. 120 of Pitt and Hocking, 1985) which they found listed in the literature on food microbiology. The present authors have devised a simple scheme (unpublished) for the rapid characterisation of yeasts associated with meat and meat products. Additionally we have attempted to link characterisation methods with isolation media which are both selective and differential (pp 12). Hopefully the next few years will see further attempts to simplify yeast identification methods for routine use in the food industry.

We have listed species according to names suggested in The Yeasts (Kreger-van Rij, 1984) and in some cases these may differ from those cited in the original paper. Appendix B provides a list of yeast names and alternatives.

D Isolation media

Over the past two decades new media have been developed for the quantitative recovery of yeasts from food in general. The earlier acidified media have slowly been replaced by those containing antibacterial agents such as oxytetracycline, rose bengal, dichloran etc (Table 1). The last two substances inhibit the radial growth of micro-fungi colonies (Rogers and Guarino, 1986) and consequently the overgrowth of yeast colonies by microfungi, a feature of acidified media, is relatively uncommon. Many studies have demonstrated that

Table 1 Examples of media used to enumerate food borne yeasts

MEDIUM		REFERENCES
Base	Additions	
	Acidified media	
Malt extract agar	10% lactic acid, final pH 3.5	Beech and Davenport (1971) Flannigan (1974)
Potato dextrose agar	10% tartaric acid, final pH 3.5	O'Toole and O'Neill (1974) Beuchat (1979)
Plate count agar (Glucose, yeast extract, casein digest)	10% citric acid, final pH 3.5	Banks and Board (1987)
Wort agar	none, pH 4.8	
	Anti-bacterial media	
Plate count agar (pH 7.0)	Chloramphenicol and chlorotetracycline+	Henson et al. (1982)
Glucose yeast extract agar (pH 7.0)	Oxytetracycline or gentamicin or both antibiotics	Mossel et al. (1970, 1975) Koburger and Rodgers (1978) Hup and Stadhouders (1972) Put and Conway (1974) Beuchat and Nail (1985)
Dextrose peptone agar (pH 7.0)	Rose Bengal chloramphenicol agar Molybdate agar containing propionate	Martin (1950) Jarvis (1973) King et al. (1979) Rale and Vakil (1984)

For a detailed discussion of all aspects of the enumeration of food-borne yeasts, see Mossel et al. (1980) and the Proceedings of the First International Workshop on "Methods for Mycologial Examination of Foods" (King et al., 1984). + For additional compounds see Davenport (1980b).

the new generation of media are generally superior to the acidified ones in terms of the number of yeasts recovered from a sample (Koburger and Rodgers, 1978). As yet, however, there is no concensus on either the best medium or incubation temperature for routine use. This is of no immediate concern because the use of any one of the newly developed media - general purpose media (Hartog 1984) - will give a general count of the yeasts contained in a food or ingredient. Such yeast counts are analogous to the total viable count of bacteria on Plate Count Agar and are helpful in assessing both the microbial quality of ingredients and the standard of hygiene at the time of production. If the yeast contribution to food spoilage is considered ecologically as advocated by Mossel (1983), then selective and differential media allowing analysis of microbial associations will be required. Many media have been devised for analysis of consortia of bacteria but, as stressed by Pitt (1988), few for yeasts other than stress-tolerant ones (Table II). A recent study at Bath University (Tudor and Board, 1989b) has shown that Violet Red Bile Glucose Agar containing oxytetracycline (VRBGO) is an excellent medium for investigating the longitudinal distribution of yeasts on equipment in broiler processing plants and the subsequent contamination of products. In addition to providing general yeast counts comparable to those given by Rose Bengal Chloramphenicol medium for example, the VRBGO allows colonies formed by the six commonly occurring genera of meat yeasts to be recognized on the basis of colour and topographical features. Another study (Dillon and Board, 1989a) has adopted a principle recommended by microbiologists working in breweries (Hartog, 1984). They included Schiffs' reagent in media to get differential counts of sulphite

Table II Examples of media used to isolate stress-tolerant and other spoilage yeasts

Medium	Purpose	Reference
Yeast Nitrogen Base NaCl (10%) + glucose^{+} (5%)	To isolate moderately xerotolerant yeasts.	Wickerham (1951)
50% glucose-yeast extract agar	To isolate xerotolerant yeasts.	Kreger-van Rij (1984)
Wort agar + sucrose (3.5%) + glucose (1.0%)	To isolate xerotolerant yeasts from foods of high salt or sugar content and for storage.	Scarr (1959)
Potato dextrose agar sucrose (60% w/v)	Isolation of *Zygosaccharomyces rouxii*	Restaino et al. (1985)
Malt extract agar + glucose (2, 20, 40 or 50% w/v)	Isolation and cultivation of xerotolerant yeasts	Pitt (1975)
Glucose-citric acid-tryptone agar	Isolation of sugar tolerant yeasts from concentrated orange juice.	Ingram (1957)
Oxoid membranes incubated on Sabouraud broth (pH 4.4)	Isolation of xerotolerant yeasts present at low concentrations	Scarr (1959)
Schwarz diagnostic medium and fuchsin sulphite	Isolation of wild yeasts from brewery plants.	Seidel (1973)

binding and non-binding yeasts in brewery samples. Dillon found that a medium containing colourless Schiffs' reagent allowed detailed studies of changes in the proportions of sulphite binding and non-binding yeasts during the storage of preserved meat and meat products.

E Manifestation of yeast spoilage

Pitt and Hocking (1985) contend that only a dozen or so yeast species (Table III) "are responsible for spoilage of foods which have been processed and packaged according to normal standards of Good Manufacturing Practices". They infer that the majority of food-borne yeasts noted in the literature are simply contaminants from natural sources. Such organisms will not grow unless some stage of a food processing operation is neglected - viz poor factory hygiene, unsatisfactory temperature control, inadequate pasteurizing temperatures/times, uncritical control of formulations, omission of preservatives etc. This Chapter lists more than 12 species of yeasts associated with the spoilage of ingredients and food. The discrepancy can be attributed to at least three factors; 1. the neglect noted above, 2. amendment of a traditional food viz dessert yoghurts (pp.74), and 3. the emergence of technologies in developing countries seeking increased revenue through value-added processing of raw materials. With the last mentioned, a novel range of adventitious contaminants could well cause spoilage until appropriate GMP was introduced, a topic discussed by Marshall (1987). Biomass formation is normally the first important event in yeast spoilage of food. From Table IV it is evident that biomass,

Table III The most commonly encountered spoilage yeasts*

Organisms	Examples of spoilage (References)
Brettanomyces intermedius+	Beer (van der Walt, 1984); wines (Barret et al., 1955); soft drinks (Sand, 1973); yoghurts (Comi et al., 1982).
Candida krusei+	Milk (Di Menna, 1956); cheese (Engel, 1986b); yoghurt (Suriyarachchi and Fleet, 1981); tomato sauce (Pitt and Richardson, 1973).
Debaryomyces hansenii+	Salt brines used in production of cured meats (Costilow et al., 1954); fermented and cured meats (Wickerham, 1957); orange juice (Put et al., 1976); milk, cream, ice-cream, cheese, yoghurt (Fleet and Mian, 1987); bread (Hartog and Kuik, 1984); seafoods (Comi et al., 1987)
Kloeckera apiculata+	Figs, tomatoes (De Camargo and Phaff, 1957); canned black cherries (Put et al., 1976); yoghurts (Spillman and Geiges, 1983).
Pichia membranaefaciens+	Olive brines (Vaughn et al., 1943); acetic acid preserves (Dakin and Day, 1958); tomato sauce (Pitt and Richardson, 1973); cheese (Choisy et al., 1986); meats (Dalton, 1984).
Rhodotorula spp.	Heat-treated apple sauce and strawberries (Put et al., 1976); cream, butter, ice-cream (Fleet and Mian, 1987); yoghurts (Spillman and Geiges, 1983); bread (Hartog and Kuik, 1984); meats (Dalton, 1984); seafoods (Comi et al., 1987).

Table III continued

Organisms	Examples of spoilage (References)
Zygosaccharomyces bailii	Fruit syrups, concentrated fruit juice (Put et al., 1976); wines (Davenport, 1981, Rankine and Pilone, 1973); tomato sauce (Pitt and Richardson, 1973); mayonnaise (Kurtzman et al., 1971); bread (Spicher, 1985).
Zygosaccharomyces bisporus	Similar capability to *Zygosacch. bailii* as a causative agent of food spoilage.
Zygosaccharomyces rouxii	Concentrated syrups, fruit juices (Tilbury, 1980); confectionery fillings (Tilbury, 1976); marzipan (Leveau and Bouix, 1979); prunes, figs (Natarajan et al. 1948); mayonnaise (Kirsop and Brocklehurst, 1982); seafoods (Sakai et al., 1983).
Saccharomyces cerevisiae	Soft drinks (Put et al., 1976); fruit juice (Röcken et al., 1981); cheese (Choisy et al., 1986); yoghurts (Green and Ibe, 1987); bread (Spicher, 1986b).
Schizosaccharomyces pombe	Sugar syrups (Pitt and Hocking, 1985);
Candida holmii+	Sauerkraut (Steinbuch, 1965); soft drinks (Pitt and Richardson, 1973); meats (Comi and Cantoni, 1985); seafoods (Comi et al., 1987).

* Based on Pitt and Hocking (1985)

+ Corresponding perfect/imperfect name given in Appendix B.

especially if pigmented, may be the sole cause of spoilage. If the presence of large yeast populations is masked by the food, then metabolic products are the major cause of spoilage. Many examples of the latter are given in Table IV. In practice the information given in this Table could aid an investigation of spoilage due to yeasts, especially if spoiled material was examined microscopically. Subsequent isolation of the causative organism on an appropriate medium would then lead to a detailed analysis of a process and the introduction of appropriate GMP.

II SUGAR-RICH INGREDIENTS AND PRODUCTS

Increasing the concentration of sugar, among other substances, in plant materials by drying is a traditional method of preservation. So too is the addition of sugar (sugaring) to products. At one time sugaring was the principal means of coping with excesses at harvest time, especially of fruit intended for out-of-season processing. Fermentation of sugar-rich products, many of which are now categorised as Intermediate Moisture Foods (Table V), is the commonest cause of spoilage. Early studies established that, almost without exception, the causative agents were stress-tolerant yeasts capable of growing, albeit slowly, in strong sugar solutions (^{o}Brix 65; equivalent to an a_w of 0.865 at 25^{o}C, Scarr and Rose. 1966, Pitt and Hocking, 1985). As noted by Ingram (1957) and again thirty years later by Rose (1987), semantics have bedevilled the choice of a descriptive term for such organisms. For the purposes of this chapter, xerotolerant (Brown, 1978) will be used in preference to the older and, until recently more commonly

Table IV Changes caused by yeast growth in foods

Change	Product	Organisms	References
Surface growth	sliced bread	*Saccharomyces fibuligera* *Pichia burtonii* *Pichia membranaefaciens* *Saccharomyces cerevisiae* *Candida humicola* *Candida parapsilosis* *Zygosaccharomyces bailii* *Torulaspora delbrueckii* *Hansenula anomala* *Geotrichum*	Spicher (1984), 1985, 1986a), Seiler (1980), Ponte & Tsen (1987)
	skinless sausages	*Candida zeylanoides* *Debaryomyces hansenii*	Tudor & Board (unpublished observations)
Surface slime with/without pigment	creamed cheese	*Sporobolomyces roseus* *Trichosporon* spp. *Candida sake* *Cryptococcus infirmo-miniatus*	Brocklehurst & Lund (1985)

Table IV continued

Change	Product	Organisms	References
Turbidity	pectin used in jam production	Not named	Olliver (1962)
Destabilization of a haze	orangeade	*Saccharomyces cerevisiae*	Röcken et al. (1981)
Gas formation			
Distortion of container	sweetened condensed milk	*Candida globosa* *Candida lactis-condensi*	Walker & Ayres (1970)
	yoghurt	*Saccharomyces cerevisiae* *Torulaspora delbrueckii* *Pichia fermentans* *Kluyveromyces marxianus* *Kloeckera apiculata* *Brettanomyces intermedius* *Candida famata* *Candida versatilis* *Candida lusitaniae* *Zygosaccharomyces ellipsoides*	Spillman & Geiges (1983)
	glacé cherries	*Zygosaccharomyces rouxii*	Tilbury (1976)

Table IV continued

Change	Product	Organisms	References
Distortion of product	easter eggs	*Zygosaccharomyces rouxii*	Tilbury (1976)
	brined cucumber	*Candida holmii* *Hansenula subpelliculosa* *Torulaspora delbrueckii* *Zygosaccharomyces rouxii*	Etchells et al. (1952)
Off-flavours	pomegranate kernels	*Hanseniaspora guilliermondii* *Metschnikowia pulcherrima* *Debaryomyces hansenii*	Juven et al. (1984)
Breakdown of plant tissues	rhubarb petioles	*Trichosporon cutaneum*	Dennis & Buhagiar (1980), Dennis (1983)

Table V Traditional IMF's susceptible to spoilage by osmophilic yeasts

Product type	Examples	Water content (% w/w)	Solute content (% w/w)	Water activity a_w
1. Syrups, sugars sweet spreads and preserves	Raw cane sugar	0.4–0.7	99.3–99.6	0.60–0.75
	Refined sucrose syrup	33.3	66.7	0.85
	Glucose or invert syrup	20.0	80.0	0.72
	Barley syrup; malt extract	20–25	75–80	0.70–0.80
	Maple syrup	26–36	64–74	0.70–0.80
	Honey, jam, marmalade	20–35	65–80	0.75–0.80
2. Fruit juice concentrates	Orange juice	35	65	0.80–0.84
	Raspberry	35	65	0.79–0.80
3. Confectionery products	Marzipan	15–17	83–85	0.75–0.80
	Glacé cherries	30	70	0.75
	Toffees and caramels	8	92	0.60–0.65
4. Bakery products	Fruit cakes	20–28	72–80	0.73–0.83
	Christmas pudding	20–25	75–80	0.70–0.77
5. Dairy products	Sweetened condensed milk	30	70	0.83
6. Dried fruits	Prunes and figs	20	80	0.68
	Dates	12–25	75–88	0.60–0.65

Table V continued

Product type	Examples	Water content (% w/w)	Solute content (% w/w)	Water activity a_w
7. Cereals	Flour, rice, pulses	16–19	81–84	0.80–0.87
	Rolled oats	10	90	0.65–0.75
8. Bread and pickled vegetables, sauces	Lactic acid fermentations: cucumbers, olives; acetic acid pickles	76–90	10–24	0.79–0.94
9. Meats	Fermented sausages, e.g. Hungarian salami, county-cured hams	74	26	0.83–0.87

(Reproduced with permission Tilbury, 1976)

used, osmophilic yeasts (Onishi 1963).

Walker and Ayres (1970) in the section on syrups in their classic review of yeast spoilage of foods were in effect reviewing problems stemming from rapid change. Namely from traditional, small scale production methods linked to local markets to those based on nascent technologies supplying national and international markets. These authors identified two complementary approaches to the control of xerotolerant yeast spoilage: the elimination where practicable of foci and routes of infection and the application of GMP at all stages of production, storage and distribution. The importance of infection foci was highlighted by Ingram (1957). He noted the presence of xerotolerant yeasts in floral nectaries. These are infected by bees and other nectarophilus insects, wasps, ants and flies. Such nectaries are therefore important sources of contamination of honey and ripe fruit. The beehive and mummified fruit are possibly the two most important niches in which xerotolerant yeasts overwinter (Davenport, 1973). Ingram also emphasized that "sugar-rich localities" were important secondary foci of infection viz the soil of apiaries and fruit plantations and the equipment and buildings used for processing sugar syrups, honey etc. Indeed before Ingram's review, Pouncy and Summers (1939) stated, "we have found osmophilic yeasts are continually introduced into a factory with every form of sugar received, including icing sugar". If the improvements suggested in the literature reviewed by Walker and Ayres (1970) and Ingram (1957) were rigorously applied then spoilage of foods preserved by sugaring ought to be a thing of the past. This view was forcibly endorsed by Pitt and Hocking

(1985). It is evident from Table VI that this ideal is not always attained. In the following discussion, examples selected from this Table will illustrate shortcomings in GMP and emphasize a point made by Ingram (1957), sugar-rich material will invariably enrich xerotolerant yeasts unless very high standards of hygiene are maintained.

A Honey

In processing nectar, bees reduce its water content from 30-60% to 15-19% and cause an appreciable conversion of sucrose through the addition of invertase as well as adding antimicrobial agents (White, 1978). There is general agreement that glucose oxidase, which produces H_2O_2 and causes a modest souring, is probably the main antimicrobial agent in honey (Bogdanov, 1984). This worker failed to substantiate claims that lysozyme plays a part in the defence of honey against infection. He did demonstrate, however, that 11 out of 12 honey samples contained a previously unknown heat and light stable antimicrobial agent, flavone pinocembrine. This is a major antimicrobial agent of propolis, a resinous material used in the repair of damaged combs and as a general disinfectant in hive hygiene. In practice these antimicrobial agents appear to be relatively ineffective in protecting honey in the hive from contamination, and from spoilage by xerotolerant yeasts (Walker and Ayres 1970). Likewise they do not prevent fermentation of harvested honey containing more than 17% moisture (White, 1978). Modern processing methods include filtration which removes extraneous materials (pollen, bits of wax) and glucose crystals as well as a

Table VI Yeasts associated with the spoilage of sugar, syrups, confectionery and concentrates

Category Product	Manifestation Spoilage	Yeasts involved	References
Natural			
Honey	Fermentation	*Zygosaccharomyces rouxii*	Walker and Ayres (1970), White (1978), Pitt and Hocking (1985)
Extracts			
Maple syrup	Fermentation	*Hansenula anomala* *"Saccharomyces behrensianus"* *Zygosaccharomyces rouxii*	Walker and Ayres (1970)
Sugar: raw cane (or beet)	Fermentation	*Zygosaccharomyces rouxii*	Scarr (1951, 1953, 1968), Scarr and Rose (1966), Tilbury (1976)
refined, crystalline	Fermentation	*Candida apicola*	Tilbury (1976)

Table VI continued

Category Product	Manifestation Spoilage	Yeasts involved	References
Syrups: white	Not stated	*Zygosaccharomyces rouxii* *Candida valida*, *Hansenula anomala* var. *anomala*, *Kloeckera apiculata* *Saccharomyces cerevisiae*	Scarr and Rose (1966), Dr R R Davenport (quoted by Tilbury, 1976)
brown	Not stated	*Zygosaccharomyces rouxii* *Zygosaccharomyces bailii* *Candida valida*, *Hansenula anomala* var. *anomala*	Observations by Dr R R Davenport (quoted by Tilbury, 1976)
in use (for ginger preparation)	Fermentation	*Zygosaccharomyces rouxii*	Brown and Lloyd (1972), Lloyd (1975a)
Sulphited	Fermentation and H_2S production	*Schizosaccharomyces pombe*	Pitt and Hocking (1985)
Molasses	Fermentation	*Zygosaccharomyces rouxii* *Saccharomyces cerevisiae* *Candida holmii*	Hall et al. (1937), Tilbury (1976, 1980)

Table VI continued

Category Product	Manifestation Spoilage	Yeasts involved	References
Malt extract	Fermentation	*Zygosaccharoromyces rouxii*	English (1953), Observations by Dr A J Reynolds (quoted by Tilbury, 1976)
Syruped			
ginger	Not stated	*Zygosaccharomyces rouxii*	Lloyd (1975a)
egg mixture	Not stated	*Hansenula subpelliculosa*	Bedford (1942)
Concentrates			
Fruit juice	Fermentation	*Zygosaccharomyces rouxii* *Zygosaccharomyces bisporus* *Zygosaccharomyces bailii*	Sand (1973), Tarkow et al. (1942)
chocolate syrup	Fermentation	*Candida etchellsii* *Candida versatilis* *Hansenula subpelliculosa*	Tilbury (1976)
Crystallised			
sugar fruits	Fermentation	*Zygosaccharomyces rouxii*	Mossel (1951)
cherries	Trapped gas exploding	*Zygosaccharomyces rouxii*	Olliver (1962, Tilbury (1976)

Table VI continued

Category Product	Manifestation Spoilage	Yeasts involved	References
Non-alcoholic beverages	Fermentation	*Saccharomyces cerevisiae* *Candida inconspicua* *Candida famata*	Put and Sand (1974)
Confectionery			
Easter eggs	Fermentation	*Zygosaccharomyces rouxii*	Tilbury (1976)
Soft centred marzipan and persipan	Fermentation	*Zygosaccharomyces rouxii* *Candida dattila*	Windisch and Neumann (1965) Blaschke-Hellmessen and Teuschel (1970), Seiler (1977, 1982)
Cake icings	Pitting and fermentation	*Zygosaccharomyces rouxii*	Seiler (1977) Pitt and Hocking (1985)
Cooked			
Jam, traditional	Gas formation	*Zygosaccharomyces rouxii*	Seiler (1977), Pitt and Hocking (1985)

Table VI continued

Category Product	Manifestation of Spoilage	Yeasts involved	References
	Fermentation	*Torulaspora delbrueckii* *Candida cantarellii* *Zygosaccharomyces bisporus*	Tilbury (1976)
low sugar	Not stated	*Zygosaccharomyces rouxii*	Horner and Anagnostopoulus (1973)
pectin	Haze	Not stated	Olliver (1962)

heat treatment having two objectives: the dissolution of minute glucose crystals and destruction of Zygosaccharomyces rouxii. This is the commonest yeast in the fermentative spoilage of honey (White, 1978). Heat-treated honey is shelf-stable unless contamination occurs during packaging and the product is then stored with inadequate temperature control. Areas of diluted honey in which Zygosacch. rouxii grow result from condensation on the lid of the container with subsequent drip.

According to Walker and Ayres (1970) these factors also lead to the spoilage of maple syrup by Zygosacch. rouxii. Mary English (1953) made a detailed study of fermenting malt extract. She demonstrated that the growth rate of this xerotolerant yeast was influenced by: the water content of the extract, inoculum size, and storage temperature. An inoculum of ca. 5.0×10^5 yeasts g^{-1} caused overt fermentation by 10 days at 30°, 35° and 37°C but by 18 days at 20° and 25°C. A small inoculum (eg. 1.5×10^3 g^{-1}) failed to initiate fermentation of malt extract at 37°C but did so by 28 days at the other incubation temperatures noted above. Despite these observations, the mass production of canned malt extract for home brewing is still associated with occasional spoilage by Zygosacch. rouxii (observations by Dr A J Reynolds quoted by Tilbury, 1976). According to Tilbury (1980) spoilage of a wide range of sugar rich commodities normally only becomes evident when counts of 10^{5-6} g^{-1} of Zygosacch. rouxii are attained.

B Sugars and syrups

A route of infection from the growing sugar-cane plant to extracted juice together with additional contamination by processing equipment has been discussed repeatedly. The grass-like leaf of sugar-cane plants tends to split longitudiually at the sheathed base (Ingram, 1957). This allows the entry of yeasts, some of which multiply in the internal spaces of plant tissues (Scarr and Rose, 1966). Yeast contamination of sugar used in the production of aguardente de cana was investigated by Shehata (1960). Samples from the sugar-cane crusher and channels taking juice to the fermenters contained Candida, Endomyces, Hansenula, Kloeckera, Saccharomyces and Schizosaccharomyces. He surmized that the yeasts were probably derived from soil adhering to sugar cane. Other sources of infection, such as plant tissue and processing equipment, were no doubt of greater importance. With the onset of fermentation, Schizosaccharomyces pombe and Saccharomyces cerevisiae became dominant. Zygosaccharomyces rouxii and Candida famata were found to be the major cause of product loss in Guyana (Tilbury, 1976). Sugar leaving the crystalizers harboured very few contaminants, infection occurred via contact with sugar residues on conveyor belts, storage bins etc. After 5 weeks storage, the yeast counts were of the order of 10^7 g^{-1} sugar. Good housekeeping did not resolve the problem; changing processing methods such that the final a_w of sugar was below 0.65 did.

Scarr (1951) isolated many yeasts from raw cane sugar imported into the UK from the Carribean, Africa and Australia. Contaminants

were confined to the film of molasses surrounding each sugar crystal. According to Scarr (1968) the contaminants died out when raw cane sugar was stored in silos having a "low RH". The nature of the process used to produce crystalline refined sugar is such that very low levels of contamination obtain (Scarr, 1963). Tilbury (1976) noted that white sugar is not normally susceptible to spoilage by xerotolerant yeasts. He did, however, record an incident where moisture migration in a silo resulted in spoilage due to Candida apicola.

Although Zygosacch. rouxii is the contaminant of raw sugars and impure sugar solutions, invertase-producing yeasts appear to be the important ones in relatively pure solutions of sucrose in water. Thus Scarr and Rose (1966) isolated the following from sugars, syrups and intermediary refinery samples: Candida apicola, Candida globosa, Candida lactis-condensi, Candida guilliermondii and Zygosacch. florentinus. An equally impressive array of yeasts (Table VI) has been isolated from (in-use?) white and brown syrups by Dr R.R. Davenport (quoted by Tilbury, 1976).

For bulk shipment of commercial liquid sugar or syrups, great care needs to be taken (Tilbury, 1976) to prevent recontamination of material sterilized by heat and filtration. Scarr (1963) advocated steam-sterilization of bulk tankers followed by effective removal of water in order to avoid "pockets of dilution" of the syrup. Sterilization of water condensing on the tanker walls can be achieved with UV lamps situated on the roof of a tanker and recontamination prevented by filtration of air entering the void

space in a tank. As with honey, maple syrup etc, condensation in commercial storage tanks must be avoided if "in-use" spoilage by xerotolerant yeasts is not to occur (Seiler, 1980). Even with rigorous GMP, problems can occur in the most carefully refined syrups especially with long term storage. Tilbury (1976) records two episodes of exploding (yeasts not named) bottles of BP syrup due to the retailer storing liquid sugar for up to six months.

Although molasses are generally microbiologically sterile, Tilbury (1976) records a case of fermentation in a ship's hold. This was associated with accidental dilution and growth of Sacch. cerevisiae and Candida holmii. Hall et al. (1937) found that Zygosaccharomyces rouxii caused fermentation of barrels of Barbados "molasses" which had been deliberately stored for several months.

C Syruped and crystallised fruit

It is evident from Table VI and the review by Walker and Ayres (1970) that Zygosacch. rouxii is the commonest cause of spoilage of fruits preserved by sugaring. Before the advent of vacuum pans with automatic recirculation and concentration of sugar, uneven distribution of sugar in glacé cherries resulted in yeast (unnamed) fermentation of relatively sugar-free zones of the fruit. Gas trapped at the sites of fermentation produced opaque patches in the finished product (Olliver, 1962). Judging from Tilbury's (1976) observations, glacé cherries produced by modern methods (presumed) also spoil through the growth of Zygosacch. rouxii, with gas production causing the fruit to explode. Pitt and Hocking (1985)

reported fermentation and H_2S production in sulphited syrups used in the manufacture of glacé fruits. Schizosaccharomyces pombe was identified as the causative organism. They also isolated this yeast from raspberry cordial (45°Brix; pH 3.0; SO_2, 250mg^{-1} kg^{-1}). A study of crystallised ginger production by Annice Lloyd (1975b) revealed a wide range (2.0 x 10^2 - 3.2 x 10^7 ml^{-1}) of contamination levels in fermenting syrups with Zygosacch. rouxii. Ginger removed from such syrups by centrifugation harboured from 1.3 x 10^2 - 6.9 x 10^5 yeasts g^{-1}. Only 24 out of 128 samples of finished product stored for up to 2 years contained viable yeasts. A large number of dead cells were evident in most samples. She recommended pasteurization for prevention of spoilage of finished product. Pieces of ginger taken from the syrup are immersed for 2 min in sucrose solution (80° Brix) at 93 ± 1°C.

D Confectionery

Unless thoroughly freed of sugar residues and then disinfected (Blaschke-Hellmessen and Teuschel,1970) equipment used in the manufacture, storage and application of jam, fudge, fondant, marshmallows, marzipan, persipan etc. will become "sugar-rich localities". The commonest spoilage yeast, Zygosacch. rouxii (Table VI), will be enriched and carried over from one production run to the next (Seiler, 1977). Growth of contaminants in ingredients may occur in stored "left overs" especially if extraneous material has been acquired during use. Thus cake crumbs in re-used marzipan provide zones of high water activity in which contaminants thrive (Seiler, 1980). Changes in manufacturing practices may also lead to

spoilage. For example, bulk delivery of jam with disruption of the pectin gel to improve dispensing, can lead to spoilage of products if pumps and pipes are not thoroughly cleansed and disinfected (Seiler, 1980). It must be stressed that the outcome of contamination of sugar-rich confectionery products will be determined by an interplay of many factors. This is evident in Fig. I in which the influence of inoculum levels and a_w on the rate of fermentation of jams is summarized. It is obvious from these data that with storage at 25°C, a small inoculum (ca. 10^2 yeasts g^{-1}) reduces the storage life of jam of a_w 0.82-0.83 to about 10 days. In practice these results mirror those of English (1953) who studied fermentation of malt extract. It also needs to be recalled that many materials used in confectionery are stored for long periods, and so an effective barrier to water uptake by hygroscopic substances such as fondant and marshmallow is essential. The encapsulation of such materials in chocolate is a traditional means of preventing water uptake (Biquet and Labuza, 1988).

III FRUITS AND VEGETABLES

Two features of Table VII need emphasis, the broad range of commodities included and the relatively large number of yeast species implicated in spoilage. As previous reviews have catalogued much of the available information (Mrak and Phaff, 1948, Dennis, 1983; Dennis and Buhagiar 1980; Walker and Ayres, 1970), this section considers ecological factors associated with yeast spoilage of some of the commodities included in Table VII. Such an approach identifies amendments in technology and improvements in GMP which

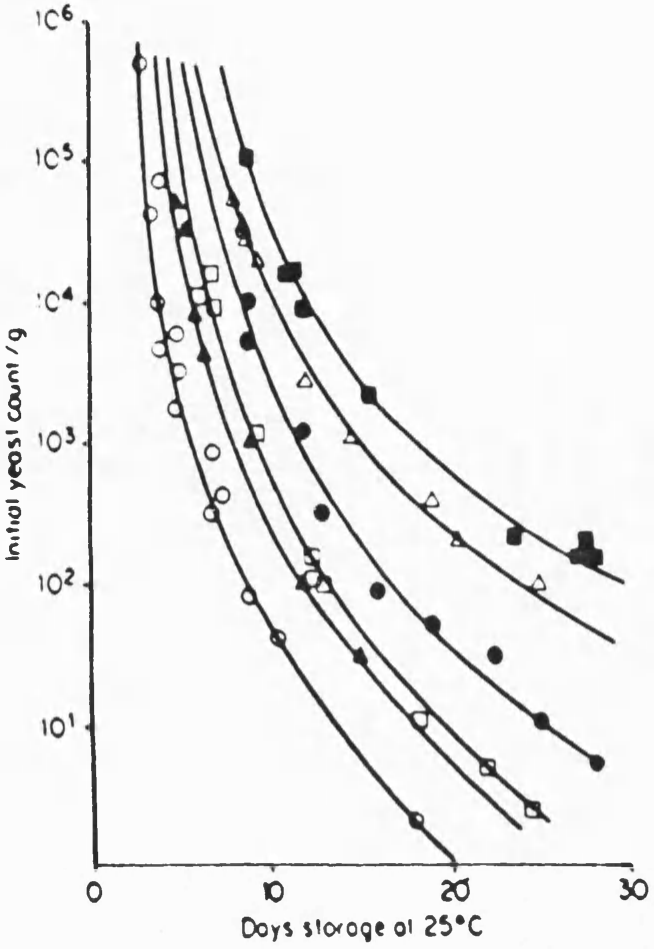


Figure 1 Effect of a_w and initial yeast inoculum level on the rate of fermentation of jam

a_w values of jams: ■ 0.73 - 0.74;
△ 0.74 - 0.75; ● 0.76 - 0.77;
□ 0.77 - 0.78; ▲ 0.78 - 0.79;
○ 0.82 - 0.83

(Reproduced with permission from the Society for Applied Bacteriology; Seiler, 1980)

Table VII Yeasts associated with the spoilage of fruit and vegetables*

Category Product	Manifestation Spoilage	Yeasts involved	References
Fresh			
Tomatoes	Fermentation	*Hanseniaspora uvarum* *Pichia kluyveri* *Kloeckera apiculata* *Candida krusei* *Nematospora coryli* *Hanseniaspora valbyensis*	De Camargo and Phaff (1957) Batra (1973)
Figs:			
Californian	Fermentation/ souring	*Candida stellata* *Kloeckera capiculata*	Mrak et al. (1942b), Miller and Phaff (1962)
		Saccharomyces cerevisiae	Phaff and Starmer (1987)
African	Souring	*Pichia membranaefaciens* *Hanseniaspora uvarum* *Kloeckera apiculata* *Candida sorboxylosa*	Lachaise (1977)
Dates:	Souring	*Candida guilliermondii*	Fellers and Clague (1942)
		Hanseniaspora valbyensis	Zein et al. (1956) Salik et al. (1979)

Table VII continued

Category Product	Manifestation Spoilage	Yeasts involved	References
Strawberries	Softening, discoloration	*Kloeckera apiculata*	Lowings (1956), Dennis and Harris (1979)
Pineapple	Soft rot	*Candida* spp.	Joarder et al. (1975)
Forced rhubarb petioles	Rotting	*Trichosporon cutaneum*	Dennis and Buhagiar (1980), Dennis (1983)
Legumes, coffee berries	Water-soaked tissue at infection site	*Nematospora coryli*	Batra (1973)
Citrus fruits	Rotting	*Nematospora* spp. *Nematospora coryli*	Ingram (1958) Batra (1973)
Comminuted			
Citrus beverage base	Fermentation	*Zygosaccharomyces bailii* var. *bailii*	Lloyd (1975b)

Table VII continued

Category Product	Manifestation Spoilage	Yeasts involved	References
Gas packed			
Pomegranate kernels	Off-flavours	*Hanseniaspora guilliermondii*, *Metschnikowia pulcherrima* *Debaryomyces hansenii*	Juven et al. (1984)
Brined/Fermented			
Sauerkraut	Discoloration - pink to grey	*Saccharomyces exiguus* *Candida krusei* *Candida* spp.	Steinbuch (1965)
Cucumbers	"Bloaters"	*Candida holmii* *Hansenula subpelliculosa;* *Torulaspora delbrueckii*, *Zygosaccharomyces rouxii*	Etchells et al. (1952)
Olives	Softening of	*Rhodotorula glutinis* var. *glutinis* *Rhodotorula minuta* var. *minuta*, *Rhodotorula rubra*	Vaughn et al. (1969)

Table VII continued

Category Product	Manifestation Spoilage	Yeasts involved	References
	Gas pocket formation (("fish-eyes")	*Saccharomyces cerevisiae* *Saccharomyces kluyveri* *Hansenula anomala* var. *anomala*	Vaughn et al. (1972)
Mushrooms	Not stated	*Hansenula anomala* var. *anomala*	Comi et al. (1981)
Japanese radish	Not stated	*Hansenula anomala* *Pichia membranaefaciens* *Candida* spp. *Rhodotorula rubra*	Kato and Nakase (1986)
Kocho	Soft, slimey surface	*Trichosporon* spp.	Gashe (1987)
Mango achaar[+]	"Blowing" of containers; Yeast growth on mango protruding above oil in container	*Candida krusei* *Kloeckera apiculata* *Hansenula subpelliculosa* *Zygosaccharomyces bailii* *Saccharomyces cerevisiae*	Van Der Riet (1982)

Table VII continued

Category Product	Manifestation Spoilage	Yeasts involved	References
Acidified			
- acetic acid			
onion, gherkins, red beetroot and red cabbage	Gas formation; rusty/sulphurous/ yeast-like odour; cream/brown sediment in clear liquor	*Zygosaccharomyces bailii* *Pichia membranaefaciens*	Dakin and Day (1958)
- acetic acid, NaCl and sugar			
cherries	Fermentation	*Zygosaccharomyces* spp.	Tilbury (1976)
Sulphited			
Strawberries	Maceration	*Trichosporon pullulans* *Cryptococcus albidus* var. *albidus*	Dennis and Buhagiar (1980) Dennis (1983)

Table VII continued

Category Product	Manifestation Spoilage	Yeasts involved	References
Dried			
Prunes Figs	White "sugar-like" coating	*Zygosaccharomyces bailii* *Zygosacch. rouxii* *Schizosaccharomyces octosporus*	Baker and Mrak (1938), Natarajan et al. (1948), Mrak et al. (1942b), Phaff et al. (1966)
Dates	Souring, off-odours. Gas pockets formed in fruit	Not stated	Fellers and Clague (1942)
	White crust	Not stated	Mrak et al. (1942a)
Frozen			
Strawberries (in 60% sucrose solution and stored at -4°C)	Not stated	*Rhodotorula* spp.	Berry and Magoon (1934)

Table VII continued

Category Product	Manifestation Spoilage	Yeasts involved	References
Cooked			
Canned/bottled cherries, raspberries strawberries apple sauce	Off-odours, gas production	*Saccharomyces cerevisiae* *Zygosaccharomyces bailii* *Kluyveromyces marxianus* var. *bulgaricus*	Put and de Jong (1980)
		Saccharomyces cerevisiae *Candida stellata* *Candida parapsilosis* *Candida kluyveri* *Pichia membranaefaciens* *Candida globosa* *Candida lactis-condensi*	Moon et al. (1985)
Processed			
Orangeade	Destabilization cloud	*Saccharomyces cerevisiae*	Röcken et al. (1981)

* For other reviews, see Mrak and Phaff (1948), Walker and Ayres (1970) and Dennis (1983).

+ For details of spoilage of Indian mano pickles (mango achaar), see Pradhan et al. (1985).

have been or need to be made in order to prevent yeast spoilage particularly of crops intended for out-of-season use. The concept of a disturbed ecosystem (Pugh and Boddy, 1988) aids interpretation of the spoilage process in fruits. In most cases such spoilage is characterised by fermentation of carbohydrates rather than the breakdown of fruit tissues. The inherent instability of man-made ecosystems (Lacey 1988 a,b), as exemplified by brined vegetables, serves this purpose in the discussion of yeast spoilage of plant materials.

A Fruits

With yeast spoilage of fruits, attention needs to be given to the flora on fruits undergoing ripening or physiological stress due to picking, packing, storage and transport (Zagory and Kader, 1988). Damage of the integument of a fruit or a structural change in a ripening fruit, as occurs in figs (see below), will inevitably make new resources - particularly carbohydrates - available to acid-tolerant micro-organisms. This situation will be exacerbated by destructive processing methods. Inadequate cleansing of processing equipment may well enrich acid-tolerant yeasts in the endogenous flora of a factory as well as those on the material being processed (Splittstoesser, 1987).

The importance of equipment in yeast transmission was demonstrated in a study of the spoilage of the petioles of forced rhubarb by a cellulolytic and xylanolytic yeast, Trichosporon cutaneum (Dennis, 1972; Stevens and Payne, 1977; Dennis and

Buhagiar, 1980). This yeast appeared to be a general contaminant (10^{4-5} g^{-1}) on most parts of forced rhubarb. Unless the knives used to cut petioles into short, uniform lengths prior to wrapping were frequently cleaned, Tr. cutaneum infected the cut-surface, and spoilage ensued with storage at room temperature. Uncritical selection of preservatives is unlikely to remedy inadequate GMP. Thus Lloyd (1975b) found that benzoate-resistant (tolerated 800 ppm under certain conditions) strains of Zygosaccharomyces bailii var. bailii were commonly responsible for spoilage of comminuted oranges or cordials prepared therefrom. Although sorbic acid proved to be a more effective preservative - 800 ppm prevented fermentation of the comminute and 400 that of a cordial - than benzoic acid, it coloured the products. She concluded that SO_2 was the only acceptable preservative.

1 Fresh fruit

Rosini et al. (1982) studied the influence of ripening on the occurrence of yeasts on grapes and the contribution of processing equipment to contamination of juice. Levels of yeast contamination varied over the surface of a grape - the highest contamination being nearest the stalk - and between grapes at various locations, the latter reflecting different rates of ripening of individual fruits. Fermentative yeasts, Kloeckera apiculata and to a lesser extent Metschnikowia pulcherrima, were not present on berries sampled 3-weeks before but they were isolated from 90% of those sampled during vintage. Saccharomyces cerevisiae was not isolated from grapes nor from juice until after the initiation of fermentation by

Kl. apiculata. Rosini and his collaborators concluded that the former was derived from equipment in the factory. A recent study (Moore et al., 1988) showed that a wider range of fermentative yeasts occurred on Arkansas white reisling grapes and in model must systems. Kl. apiculata and its perfect form, Hanseniaspora uvarum, were the commonest fermentative organisms among the 873 yeasts (representing 16 genera) which Kamra and Madan (1987) isolated from 540 samples of fleshy fruits. Buhagiar and Barnett (1971) identified 29 of the 956 strains of yeasts isolated from strawberries obtained from a Norfolk farm with Kl. apiculata. In an earlier study of strawberries obtained from 4 sources in Kent, Lowings (1956) found that Kl. apiculata developed "abundantly" on injured fruits. With 10-14 days storage at 25°C, a cream-coloured mass of this yeast developed in the inoculation wound which enlarged to form a crater. Dennis (1983) and Dennis and Buhagiar (1980) stress that they have never observed the growth of this yeast during extensive studies of whole as well as naturally-injured strawberries.

Sporadic cases of disintegration have been noted in sulphited strawberries intended for out-of-season jam making (Dennis and Harris, 1979; Harris and Dennis, 1979; Dennis et al, 1979; Archer, 1976; Archer and Fielding, 1979). Although polygalacturonase produced by Mucor and Rhizopus spp. are considered to be the commonest cause of disintegration (Dennis et al., 1979), sulphite-stable pectolytic enzymes (Dennis and Harris, 1979) are produced by yeasts associated with freshly picked strawberries viz Trichosporon pullulans and Cryptococcus albidus var. albidus.

Buhagiar and Barnett (1971) identified 424 of their 956 strawberry isolates with the latter species. According to Dennis and Buhagiar (1980) there has only been one authentic report of strawberry maceration attributable to yeast infection. A soft rot in harvested pineapples has been attributed to infection with Candida spp. even though no evidence of the production of cell-wall degrading enzymes was given (Joarder et al., 1975).

In contrast with the vague association of yeast with the spoilage of strawberries, there is no doubt that such an association exists in the fermentative spoilage of tomatoes and figs in California. De Camargo and Phaff (1957) demonstrated the presence of the following yeasts, Hanseniaspora uvarum, Kloeckera apiculata and Pichia kluyveri. These species were also dominant in the alimentary canal of Drosophila melanogaster which oviposits in cracked or damaged tomatoes. The authors concluded that fruit flies were important vectors in spreading fermentative yeasts through fields of tomatoes. They also surmised that such yeasts were brought to tomato crops by flies migrating from peach and prune orchards and vineyards following harvesting. A more complex involvement of insects with yeast spoilage is evident in the fermentation/souring of Smyrna-type figs in California. Like all figs, the Smyrna variety is a synconium but its hollow receptacle is lined only with pistillate flowers. Pollination is brought about by insects taking pollen through the 'eye' in the hollow receptacle, an aperture closed by overlapping scales in immature fruits (Mrak et al., 1942b). Caprifigs, an inedible variety, produce both staminate and pistillate florets in the first (profichi) of the 3 crops

produced per annum. Pollination of all three crops is mediated by Blastophaga wasps which live within figs. To achieve pollination of Smyrna figs, perforated bags containing profichi caprofigs are hung in Smyrna trees. The fig wasps not only introduce pollen into the hollow receptacle of the Smyrna figs but also a yeast, Candida guilliermondi var. carpophila, and a bacterium, Serratia ficaria. Both organisms grow without producing overt symptoms of spoilage (Miller and Phaff, 1962). It has been surmised (Phaff and Starmer, 1987) that yeast growth results in an attractant for drosophilae seeking a niche for egg laying. On entering the "eye" of the hollow receptacle, which at this time is lined with "juicey" florets (Mrak et al., 1942b), the fruit flies introduce yeasts (Hanseniaspora valbyensis, H' spora. uvarum, Kl. apiculata and Candida stellata) which bring about an alcoholic fermentation of sugars. As spoilage developes liquid, having a vinegar-like odour because of ethanol oxidation by acetic acid bacteria, drips from the "eye" of the fruit (Mrak et al.,1942b; Miller and Phaff, 1962). Figs on the orchard floor are also an important depot of infection; dried-fruit beetles (Caprophilus hemipterus) transfer yeasts from this source to sound figs on the tree. A microbial succession also occurs in endemic African figs (Lachaise, 1977). Candida fructus colonizes the immature receptacle which is also a breeding site for Lissocephala spp. (Drosophilidae). On maturation the exocarp of the synconium is infected with Pichia membranaefaciens, Hanseniaspora valbyensis, H' spora. uvarum, Kl. apiculata, Candida sorboxylosa as well as acetic acid bacteria and fermentation/souring ensues. As fruit flies use the rotting exocarp as a food source and breeding site, opportunities are readily available for the transfer of spoilage

organisms from infected to sound fruit. Fellers and Clague (1942) noted that some dates sour on the farm in California and they surmised that insects spread infection from sour to fresh fruit. The yeasts associated with the souring of dates are listed in Table VII.

Fresh food, especially meat, packed in sealed containers with modified atmospheres has become a feature of European supermarkets (Young et al., 1988). The composition of the trapped atmosphere has a profound elective action on contaminants on the product (Lioutas, 1988). Two recent studies suggest that yeast growth leading to spoilage could become a problem with some but not all varieties of fruit packed in this manner. Abbey et al. (1988) isolated Rhodotorula, Pichia and Cryptococcus spp. from the skin of whole watermelon. Yeasts were not isolated from juice expressed from unwrapped slices of melon stored for 8 days at $5^{o}C$. In contrast Pichia norvegensis and Cryptococcus laurentii were present in very small microbial populations (2.5×10^{2} ml^{-1}) in the juice obtained from wrapped (aluminium foil) slices of water melon stored at $5^{o}C$. Neither yeast was implicated with the undesirable organoleptic changes which developed with storage at $25^{o}C$, even though both contributed significantly to the very large populations in juice expressed after 2 days, 2.2×10^{8} organisms ml^{-1} in wrapped and 1,5 $\times 10^{6}$ ml^{-1} in unwrapped slices of watermelon. Juven et al. (1984) studied changes in the size of the populations of yeasts and Acetobacter and Gluconobacter in the edible part (kernel) of pomegranates placed on polystyrene trays, overwrapped with a film having low transmission rates for O_2 and CO_2 and flushed with N_2.

There was no appreciable change in the yeast and mould counts (log_{10}) - ca 3.2 - 3.4 g^{-1}, or those of bacteria - 3.1-3.3 g^{-1} - on kernels stored at 1°C. At 5°C the bacteria as well as the yeast and mould populations peaked at 10-15 days. The subsequent decline in the populations was attributed to the accumulation of CO_2 in the packs, 20% of the trapped gases with storage at 1° and 34.5% at 5°C. Strains of Hanseniaspora guilliermondii, Metschnikowia pulcherrima and Debaryomyces hansenii as well as acetic acid bacteria produced off-flavours in pomegranate juice. Even so, the authors concluded that "the yeasts are likely to produce more significant undesirable changes in the product than are the acetic acid bacteria". It would seem reasonable to reserve judgement on the actual contributions of bacteria and yeasts to spoilage of pomegranate kernels stored in this manner until studies with mixed cultures in pomegranate juice are done. The available evidence does suggest the possibility of alcoholic fermentation linked with souring due to ethanol oxidation by acetic acid bacteria, a situation which has been identified in fig spoilage (see above).

The current trend of reducing the salt content of foods has led to yeast spoilage of daikon (Raphinus sativus) intended for the production of takuan-zuke (Kato and Nakase, 1986). Hansenula anomala, P. membranaefaciens, Candida spp. and Rhodotorula rubra were considered to be spoilage organisms even though Saccharomyces servazzii was the dominant organism in nitrogen-packed product and Deb. hansenii in that exposed to a normal atmosphere. Spoilage problems were overcome by nitrogen packaging and the use of a brine (ca. 6% NaCl; pH 4.3) containing 2% ethanol as a consequence of

yeast fermentation.

2 Dried Fruits

Drying is a very old method of fruit preservation. Its success depends upon the drying method, traditionally insolation, producing a commodity having a low a_w as a consequence of a decreased water content and an increased concentration of solutes (Table V). With prunes, for example, a water content of ca. 12/15-22/25% ensures stability against microbial attack (Fellers and Clague, 1942; Pitt and Christian, 1968). Needless to say stability is only assured when water uptake by dried fruits is prevented during storage. In his review of the commercial and technological evolution of the dried fruit industry in California, Mrak (1941) identified many factors which led to the fermentation of sun-dried prunes, figs and natural as well as soda-dipped raisins etc. The following were considered to be of particular importance: mechanical damage of fruit at harvest or during processing, heavy rains during the drying season, and the use of immature fruits or those that were fermenting at harvest time. The last feature was shown to be important in the spoilage of figs (Natarajan et al., 1948) and dates (Fellers and Clague, 1942). The latter authors also noted that some date varieties were particularly prone to souring whereas others never soured. Mrak (1941) concluded that "if fresh fruit is clean and free from infection when introduced into a dehydrater, it should be in a similar condition when removed.... This is not always true when fruit is dried in the sun".

The literature on the spoilage of dried fruits reviewed by Walker and Ayres (1970) was derived in the main from studies of sun-dried material. It is evident in their review as well as from the summary in Table VII that drying selects osmotolerant yeasts. Spoilage was commonly manifested by souring, off-odours and/or gas production. In some instances the gas remained trapped in pockets in fermenting fruits, a situation analogous to that found in glacé cherries by Olliver (1962). Baker and Mrak (1938) investigated the development of a white coating ("sugaring") on prunes and figs during and occassionally after processing; they noted that the coating was formed from yeast cells and sugar crystals. According to Pitt and Hocking (1985) the coating is not generally regarded as spoilage even though it is the product of the cells of Zygosaccharomyces rouxii acting as crystallisation nuclei. The storage stability of dried fruits can be compromised by modern packaging methods. Fermentation of "high-moisture" prunes (35% water cf. 15-22% of traditional material) may lead to distortion of the packs unless the fruit is pasteurised or treated with a preservative (Bolin et al., 1972; McBean and Pitt, 1965). Attempts in Crete to package crystallised citrus peel in sealed containers were initially thwarted because packs were distorted by gas probably of yeast origin (Dr. G.J. Nychas, pers. comm.).

B Vegetables

1 Brined Vegetables

Yeast spoilage of the main groups of brined vegetables must be

considered in the context of the fermentation which is intended to prolong storage life. The inherent instability of man-made ecosystems has been exploited over thousands of years to produce conditions conducive to the long term preservation of a diverse range of plant products. Cabbage (eg. sauerkraut), mixtures of vegetables (eg. paw-tsay, kimchi), fruits (eg. olives, cucumbers) or ginger (Brown and Lloyd, 1972) are mixed with salt, either as crystals or brine, and left to ferment. Fleming (1982) discussed the various technologies adopted for commercial use.

The salt extracts cell sap which supports the growth of a succession of micro-organisms (Daeschel et al., 1988) providing the plant materials do not contain inhibitors. Green olives (Fleming and Etchells, 1967) contain oleuropein which inhibits lactic acid bacteria. The following sequence of bacteria commonly occurs in the fermentation of sauerkraut (Pedersen, 1960; Steinkraus, 1983) and Kimchi (Chun, 1981): Gram-negative aerobes and facultative anaerobes Enterococcus spp., Leuconostoc mesenteroides, Lactobacillus brevis, Pediococcus pentosaceus and Lactobacillus plantarum. In practice many factors, especially attributes of the vegetables, salt concentration, temperature and the buffering capacity of the brine, will determine the duration - and hence the extent - of the contribution of the various species of lactic acid bacteria to fermentation. A successful fermentation results from the sequential selection of members of the lactic acid bacteria group as a consequence of the progressive accumulation of lactic acid and to a lesser extent, acetic acid. Eventually even the most acid-tolerant organisms are inhibited. The extensive literature on fermented

plant materials identifies Lactobacillus plantarum as the terminal bacterium of the succession. Recently, however, a previously unknown organism, Lactobacillus graminis, was found to be the most acid tolerant member of the succession in silage (Beck et al., 1988).

According to Fleming (1982) yeasts are responsible for a "secondary fermentation" - an unfortunate choice of term for a dynamic situation subject to many variables - which occurs concurrently with the bacterial fermentation or subsequently if fermentable carbohydrates are present at the time of acid-inhibition of bacteria. Comprehensive studies of cucumber brines showed that the following fermentative yeasts occurred (Etchells and Bell, 1950a,b; Etchells et al., 1952, 1953, 1961): Candida versatilis, Hansenula subpelliculosa, Candida lactis-condensi, Candida holmii, Torulaspora delbrueckii, Zygosaccharomyces bailii, Candida etchellsii and Hansenula anomala. Etchells et al. (1952), who investigated yeast contamination of cucumber brines (4-18% NaCl; pH 3.1-4.8) in Indiana, Michigan and Wisconsin, USA, noted a succession during fermentation. Candida holmii tended to be the predominant yeast in the first 2-30 days of fermentation. Torulaspora delbrueckii, Hansenula subpelliculosa and Zygosaccharomyces rouxii were active in the intermediate stage, and Candida versatilis in the 70 - 110 days of fermentation. Indeed the last mentioned was isolated from brines stored for 12 -14 months. The fermentation of Manzanillo olives can be dominated by yeasts, a condition known as a "stuck" fermentation (Vaughn et al., 1943). This variety of olive is naturally deficient in fermentable

carbohydrates. Much of that in freshly-picked fruit is lost during the lye treatment and subsequent washing procedures. Addition of sugar merely exacerbates the problem; it is remedied by draining off the abnormal brine and replacing it with one that has been heavily inoculated with a normal, actively fermenting brine.

Green ginger rhizomes stored in acidified (pH 3.0-3.5) brine (7-9%) did not support the growth of lactic acid bacteria (Brown and Lloyd, 1972). A vigourous fermentation occurred in the first sixteen days of storage (Lloyd, 1975c). The following yeasts were isolated (numbers in parenthesis): Candida tropicalis (15), C. rugosa (6), C. krusei (3), Pichia ohmeri (19), C. membranaefaciens (2), Hansenula anomola (11) and Rhodotorula rubra (1). Candida spp. dominated the initial fermentation whereas the much more salt tolerant Pichia ohmeri was predominant at the end of fermentation. Brown and Lloyd (1972) concluded that there was no obvious advantage to be gained from yeast fermentation of ginger. Indeed loss of product, a darkening of ginger and undesirable changes in flavour and texture were avoided when yeast growth in stored ginger was prevented by sulphite.

Fleming (1982) states that yeasts are of little importance in the fermentation of sauerkraut because low levels of salt do not favour their selection. With pickled cauliflower (dry salting), the claim has been made that mustard contributes to preservation as a consequence of the growth of lactic acid bacteria being stimulated at the expense of yeasts ("mycoderma"), which cause spoilage by growing on the exposed surface of the product (Sethi and Anand,

1984). These authors cited several references to this phenomenon occurring in pickled cabbage, turnip and cucumber and concluded that "mustard showed a selective preservative action". Future studies may well show that stimulation of the growth of lactic acid bacteria may be due to the addition of trace metals, particularly Mn^{2+} (Zaika and Kissinger, 1984), rather than the presence of an antimycotic in mustard. Kloeckera apiculata, Hansenula subpelliculosa, Zygosaccharomyces bailii and Saccharomyces cerevisiae have been associated with fermentative spoilage during the maturation phase of green mango achaar, a mixture of slices of green mango, salt and spices (Van Der Riet, 1982). The growth of Candida krusei on mango projecting above a layer of oil on the finished product is also considered to be a spoilage problem. Pradhan et al. (1985) studied spoiled mango pickles (21 samples) obtained from homes in Pune, India. The salt content of the samples ranged from 2.8-17.13%. Sixteen yeast strains were isolated and members of the following genera identified: Saccharomyces, Hansenula, Kloeckera and Pichia.

The literature presents an equivocal picture on the contribution of yeast growth on the more subtle organoleptic properties of pickled vegetables. Thus Chao (1949), who studied the fermentation of paw-tsay, concluded that "yeast is a common contaminant, which, when not forming a thick film of mycoderma, is considered harmless". Suzuki et al. (1987), who isolated 80 yeasts from 39 samples of 29 types of fermented foods in Thailand, were of the opinion that these organisms play an important role in the production and ripening of a range of products. Regardless of the extent of the relative contributions of bacteria and yeasts to the

acidification of brined vegetables, long term preservation is dependent upon conservation of organic acids. This calls for an air-tight fermentation vessel (Fig. 2) to preclude the growth of oxidative yeasts. Such yeasts are of common occurrence in cucumber brines (Fleming, 1982) viz. Debaryomyces hansenii, Pichia ohmeri, Zygosaccharomyces rouxii, Candida krusei and Rhodotorula spp.

The following are the important physiological attributes of yeasts associated with the spoilage of brined vegetables: gas production from carbohydrates, pigment production and synthesis of pectinolytic or other enzymes that macerate plant material.

An example of spoilage due to gas production, a "stuck" fermentation, was discussed above. Production of gas and turbidity in a brine can be caused by yeasts (unnamed) growing in a commodity from which the carbohydrates were not completely removed during the fermentation stage (Fleming, 1982). The most important gas-induced spoilage problem occurs in fermented cucumbers. Fleming and Pharr (1980) and Corey et al. (1983) observed that liquid clogging the intracellular gas spaces in the tissues of brined cucumbers hampered gaseous diffusion. Nitrogen, the dominant gas in the tissues, diffused slowly from the fruit. Carbon dioxide on the other hand, rapidly diffused into the cucumber and eventually produced a pressure that caused gas pockets ("hollow stock") in and inflation of the fruit ("bloaters"). Organisms that produce CO_2 during fermentation, such as the fermentative yeasts listed above, have been implicated (Etchells and Bell, 1950a) in this form of spoilage. With the advent of nitrogen-purging of fermenting cucumber brines

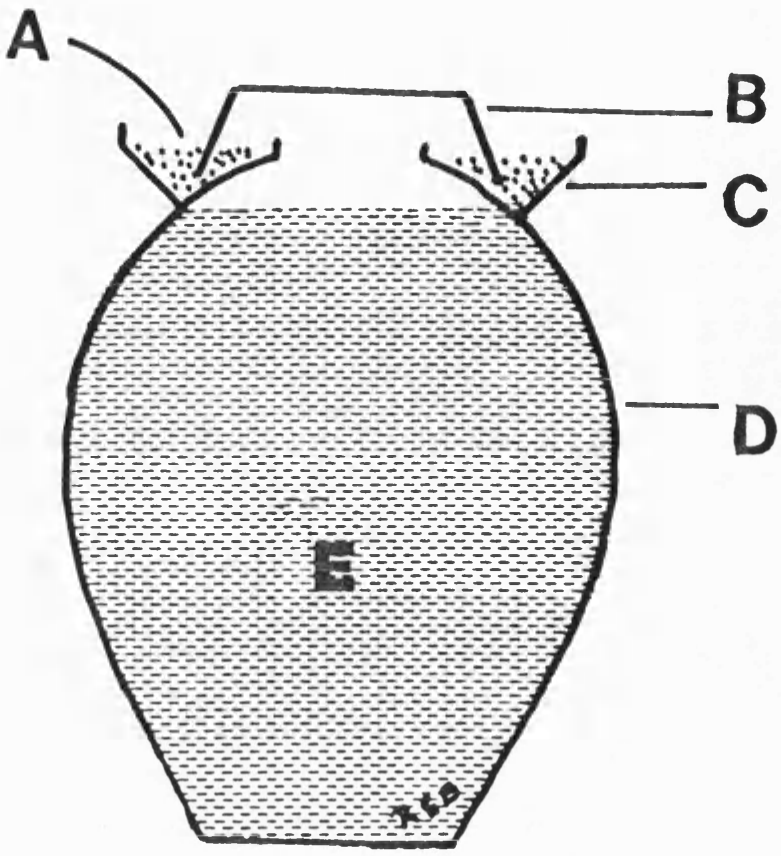


Figure 2 Longitudinal diagram of a vessel used for the fermentation of Paw-Tsay (Chao, 1949)

A Water-seal

B Cover

C Gutter

D Jar

E Vegetables in brine

(Fleming, 1982), "bloaters" ought to be a thing of the past. Indeed the introduction of this technique has led to studies (Daeschel et al., 1988) of mixed starter cultures, eg. Saccharomyces cerevisiae and Lactobacillus plantarum. Their use could well reduce the time to achieve a complete fermentation of all the fermentable carbohydrate. Apart from a saving in purging costs, the absence of carbohydrates would preclude subsequent yeast spoilage of packaged material. Additionally, a mixed culture offers an opportunity of producing low-acid products which may have desirable flavours due to yeast growth. "Fish-eye" spoilage of olives was recorded by Vaughn et al. (1972) and the following fermentative yeasts implicated: Saccharomyces cerevisiae, Saccharomyces kluyveri and Hansenula anomala var. anomala. The problem arose from a manufacturer reducing the salt content of a brine and (possibly) using a mineral rather than lactic and acetic acids as an acidulant. These conditions led to rapid yeast growth with formation of gas pockets in the fruit.

Walker and Ayres (1970) cited the reports of many workers who had examined outbreaks of pink sauerkraut. Oxidative yeasts (unnamed) were implicated in such episodes and there appeared to be general agreement that yeast growth had been stimulated by high temperatures, high salt concentrations, exposure of kraut to air or the trapping of air in shredded cabbage. Steinbuch (1965) was unable to precisely identify factors leading to discoloration of sauerkraut by Saccharomyces exiguus. He claimed that such problems were not resolved by thorough cleansing of processing equipment.

Softening of fermented plant material is occassionally due to yeasts. Vaughn et al. (1969) reported the production of polygalacturonases by the following yeasts which grew on the surface of brine and caused softening of Spanish-style green olives: Rhodotorula glutinis var. glutinis, Rhodotorula minuta var. minuta and Rhodotorula rubra. In another study Vaughn and his co-workers (1969) isolated Saccharomyces cerevisiae and Sacch. kluyveri from olives which had become soft during storage in a brine of low salt content (3-4%). Both yeasts produced pectin esterases and polygalacturonases but not pectic acid trans-eliminase. Polygalacturonase (but not pectinesterase nor pectinlyase) was isolated from cultures of Saccharomyces associated with the impairment of cloud stability of orangeade (Röcken et al., 1981). Kocho, a fermented product formed from pulverised Ensette ventricosum, is wrapped in ensette leaves and stored in a leaf-lined pit in the ground. If anaerobic conditions are not maintained in the pit, the surface of kocho becomes soft, slimy and discolored. Gashe (1987) attributed these changes to a Trichosporon spp. It would be of interest to establish whether or not this isolate produced cellulolytic and xylanolytic enzymes as do those of Trichosporon cutaneum associated with spoilage of the petioles of forced rhubarb (Dennis and Buhagiar, 1980).

2 Pickled Vegetables

Zygosaccharomyces bailii and, to a much lesser extent, Pichia membranaefaciens have been reported (Dakin and Day, 1958) to cause spoilage of acetic acid preserves such as pickled onions, beetroot,

red cabbage and piccalilli. Spoilage was manifested by gas formation accompanied by a sulphurous or yeast-like off-odour. In preserves with a clear liquid phase, creamy-brown sediments were formed. Bell and Etchells (1952) isolated yeasts closely related to Torulaspora delbrueckii from 15 spoiled (gas formed) samples of sweet pickles (cucumbers in a solution of sucrose in vinegar) made by 3 manufacturers.

3 Canned and bottled fruit

According to Dennis (1983) the spoilage of heated fruit products due to formation of gas and off-odour by the yeasts listed in Table VII is due to underprocessing. The possible contribution of heat-resistant ascopores to this problem has been discussed by Put and De Jong (1980). Even with adequate processing, leaker-spoilage due to yeast transfer across the seams in cans at the time of post-processing cooling must not be overlooked (Put et al., 1980).

4 Ready to use salads

A subsequent discussion (pp. 86) draws attention to the complex interactions occurring between components of mayonnaise-based (dressed) salads. The nature of the eventual association of spoilage organisms determined both the duration of acceptable shelf-life and the changes in organoleptic attributes. Equally complex but as yet poorly defined ecosystems are created by wrapping ready-to-use (undressed) salad vegetables in flexible films having

different rates of diffusion for oxygen and CO_2. Investigations of some of the earliest products packed in this manner (eg. Shapiro and Holder, 1960) revealed that the size of the microbial populations on cut salad-greens were markedly increased by "elaborate" handling during preparation. Bacteria, especially Pseudomonas and Erwinia spp. of soil origin, were the dominant contaminants. This situation obtains today (Brocklehurst et al., 1987; Buick and Damoglou, 1987; Manvell and Ackland, 1986; Carlin et al., 1989). Lactobacillus spp. were important contaminants of stored salads (Brocklehurst et al., 1987) especially those in vacuum packs (Buick and Damoglou, 1987) and those subjected to temperature abuse during storage (Manvell and Acklund, 1986). In one study (Brocklehurst et al., 1987) the populations of yeasts (4.3 - 7.9g^{-1}) probably formed a biomass that was little different from that of the lactic acid bacteria (5.6 - 8.9 g^{-1}). In another (Carlin et al., 1989), the populations of these two groups of organisms, as judged by cfu's, were similar (ca. 1.0 x 10^6 cfu g^{-1}), from the 7 - 14th days of storage (10^{o}C)of grated carrots. These workers concluded that the lactic acid bacteria were the main spoilage organisms. In view of the comparable sizes of the bacteria and yeast populations and a possible synergism (eg. Mossel and Ingram, 1955) between these two groups, a final judgement on yeast spoilage of undressed salads ought to be deferred until future studies have probed the complex ecosystems of such products in greater detail.

IV MILK AND DAIRY PRODUCTS

Fresh milk (pH neutral) is highly perishable even though it

contains the antimicrobial agents, agglutinins, lactoferrin, lysozyme and the lactoperoxidase system (Reiter, 1985). The nature of spoilage of unpasteurized milk has been profoundly affected by changes in farm practices and the methods used to transport milk to dairies in developed countries. Souring due to lactose fermentation by lactic acid bacteria, especially Lactococcus raffinolactis, occurred before milk was adequately cooled and kept chilled on the farm and during transport to the dairy. Today a cold chain from the farm via the dairy to the consumer has led to psychrotrophic bacteria - eg. Pseudomonas fragi - dominating the flora of unpasteurized and their thermostable lipases and proteinases persisting in pasteurized milk (Law and Mabbitt, 1983). These non-lactose fermenting psychrotrophs do not aid the growth of yeast because the milk does not sour.

As far as can be ascertained yeasts rarely if ever cause spoilage of milk per se. Although Walker and Ayres (1970) catalogued many examples of yeast spoilage of dairy products, recent reviews of dairy microbiology (Cousin, 1982; Law and Mabbitt, 1983) pay scant attention to these organisms. A presumption that "traditional" problems (Walker and Ayres, 1970) associated with yeasts have been eliminated by the cold chain noted above, pasteurization and the adoption of GMP throughout the dairy industries of developed countries is not endorsed by recent studies. A summary of several surveys (Table VIII) reveals: (A) that Cryptococcus spp., Candida diffluens and especially C. famata are common contaminants of pasteurized milk, (B) that Rhodotorula glutinis and Rh. rubra are associated with products based on milk

Table VIII The occurrence of yeasts in milk and milk products

Genera species	PRODUCT															
	Milk Raw			Milk Pasteurised	Cream	Butter	Ice Cream	Cheese		Curd cheese	"Creamed cheese"	Hard cheese	Yoghurt			
	1*	2	2	3	3	3	3	3	4	5	6	7	3	8	9	10
	27°C	RT	37°C	25°C	25°C	25°C	25°C	25°C	NS		7/20°C		25°C	30°C	25°C	NS
Candida albicans	-	-	-	-	-	-	-	-	-	-	-	2[b]	-	-	-	-
blankii	-	-	-	8[b]	-	-	-	-	-	-	-	-	-	-	-	-
curvata	38[a]	17[a]	-	-	-	-	-	-	-	2.3[b]	-	-	-	-	-	-
diffluens	-	-	-	8	14	6	-	14	-	-	-	-	17[b]	-	-	-
famata	11	-	-	27	24	-	27	38	-	6.4	!	4	41	-	21	-
guilliermondii	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	+
kefyr	-	-	8[a]	-	-	-	-	-	-	14	-	-	-	-	-	+
krusei	2	10	38	-	-	-	-	-	-	5.2	-	-	-	51[b]	-	+
inconspicua	-	-	-	-	-	-	-	-	-	-	10	-	-	-	-	-
lambica	1	-	-	-	-	-	-	-	-	3.4	-	-	-	-	-	-
lipolytica	-	-	-	-	-	-	-	-	-	8.6	+	5	-	-	-	-
lusitaniae	-	-	-	-	-	-	-	-	-	-	-	-	-	65	-	-
vini	-	-	22	-	-	-	-	-	-	-	-	-	-	-	-	+
pinus	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-
parapsilosis	-	-	9	-	-	-	-	-	-	-	-	-	-	-	-	-
rugosa	-	8	-	-	-	-	-	-	-	-	-	-		4	-	-
sake	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
stellata	-	-	-	-	-	-	-	-	-	-		-	15	-	-	-
versatilis	-	-	-	-	-	-	-	-	14[c]	-	-	-	-	-	2	-
valida	-	-	-	-	-	-	-	-	-	14	-	6	-	-	-	-
zeylanoides	-	9	-	-	-	-	-	-	-	2.9	+	-	-	-	-	-
Brettanomyces intermedius	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cryptococcus albidus	-	14	-	-	-	-	13	-	-	-	+	-	-	-	-	-
flavus	-	-	-	12	-	-	7	8	-	-	-	-	-	-	-	-
laurentii	-	14	-	-	9	6	-	-	-	-	+	-	-	-	-	-
infirmo-miniatus	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Debaryomyces hansenii	-	-	-	-	-	-	-	-	248	-	-	-	-	-	2	-
Hansenula anomala	-	-	-	-	-	-	-	-	12	-	-	-	-	-	-	-

Table VIII continued

Genera species	PRODUCT															
	Milk Raw			Milk Pasteurised	Cream	Butter	Ice Cream	Cheese		Curd cheese	"Creamed cheese"	Hard cheese	Yoghurt			
	1*	2	2	3	3	3	3	3	4	5	6	7	3	8	9	10
	27°C	RT	37°C	25°C	25°C	25°C	25°C	25°C	NS		7/20°C		25°C	30°C	25°C	NS
Kluyveromyces																
marxianus	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
var. *bulgaricus*	-	-	-	-	-	-	-	-	14	-	-	-	-	-	-	-
var. *marxianus*	-	-	-	20	-	-	-	19	79	18	-	-	24	48	11	-
Pichia																
burtonii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
farinosa	-	-	-	-	-	-	-	-	-	-	-	14	-	-	-	-
fermentans	-	-	8	-	-	-	-	-	16	-	-	-	-	-	-	-
kluyvera	-	-	-	-	-	-	-	-	6	-	-	-	-	-	-	-
membranaefaciens	-	-	-	-	-	-	-	-	28	6.9	-	-	-	-	-	-
toletana	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
Rhodotorula																
glutinis	-	-	-	-	19	25	20	-	-	-	-	-	-	-	-	-
rubra	-	6	-	-	5	12	13	-	-	-	-	-	-	-	6	+
minuta	-	-	-	-	-	-	-	-	-	-	-	9	-	-	-	-
Saccharomyces																
cerevisiae	-	-	5	-	-	-	-	8	106	4.2	-	-	29	6	9	-
Sporobolomyces																
roseus	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Saccharomycopsis																
lipolytica	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Trichosporon	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
pullulans	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
cutaneum	17	-	-	-	-	-	-	-	-	5.2	-	-	-	-	-	-

Notes to Table VIII

1. Engel (1986a) - a, % of isolate (296) obtained from 128 samples; 2. Di Menna (1956) - a, % of isolates identified; 265 isolates with incubation at 37°C and 157 at room temperature (RT). 3. Fleet and Mian (1987) - b, percent of samples in which named yeasts occurred, 161 samples. 4. Choisy et al. (1986) - c, number of isolates from a range of cheeses; 858 isolates in total; 5. Engel (1986b) - b, percent of samples (317) of fresh cheese - mainly German sour skim milk curd cheese - in which named yeasts occurred. 6. Brocklehurst and Lund (1985) - +, organism isolated from creamed cottage cheese alone or containing (i) peppers and onions, (ii) prawns, (iii) salmon and cucumber or (iv) cheddar cheese and onions from various manufacturers; 4 tubs per consignment being examined at each sampling interval during storage at 7°C with a total of 36 tubs being examined in total. 7. El-Essawy et al. (1984) - b % of samples (56) of hard cheese (Roumy) containing named organism. 8. Green and Ibe (1987) - b, percent of samples (100) in which named yeasts occurred in Nigeria; 100 samples. 9. Suriyarachchi and Fleet (1981) - general survey (128 samples) Australia. 10. Surveys conducted by Soulides (1956) and Sousa and Uden (quoted by Rašić and Kurmann, 1978) and Comi et al. (1982) - *, occurrence of named isolates. *Incubation temperature; NS, not stated, + Machota et al. (1987) isolated yeasts from 95% of the 103 samples taken from a tank at the Madrid milking cooperative between October 1981 and January 1983; Trichosporon was the most commonly isolated genus. Members of this genus were isolated from 32 samples of milk by Bridge-Cooke and Brazis (1968). According to Salji et al. (1988) "moulds, yeasts and psychtrotrophs were instrumental in cutting down the shelf-life" of pasteurised fresh milk manufactured in Saudi Arabia. The milk obtained from one plant, for example, contained 10 cfu moulds and yeasts ml^{-1} on the day of production but 9.2×10^4 ml^{-1} after 10 days at 7°C.

fats, and (C) that representatives of 12 genera of yeasts occur in curd cheese, creamed cheese (with and without non-dairy ingredients) and yoghurts.

Representatives of only three of the 12 genera noted in Table VIII were isolated in a recent survey of pasteurized milk (Fleet and Mian, 1987). There are at least 3 ways of interpreting this discrepancy: (1) the techniques used to survey pasteurized milk were not sufficiently sensitive to detect the presence of yeasts occurring in very small numbers, (2) equipment is an important source of yeast contaminants in products such as cheese that undergo complex manufacturing processes, and (3) the use of non-dairy ingredients introduces yeasts to dairy products. The hands of dairy workers may also be a source of yeast contamination. The following were isolated from those of workers in a milk processing plant (Caramello et al., 1987): Candida humicola, C. parapsilosis, C. guilliermondii, C. albicans and C. zeylanoides.

A Creams

Walker and Ayres (1970) surmised that products "containing some acid" were vulnerable to yeast growth. This was a particular problem, especially during the summer months, with cream intended for butter production. Growth of Candida kefyr, Kl. marxianus var. lactis, both of which ferment lactose, led to off-flavours and gas production ("foamy cream"). The latter attribute was associated with cream in which other organisms, presumably lactic acid bacteria, caused acid-coagulation of casein. According to Davis

(1971) yeast spoilage is now uncommon in creams which are commercially produced though it still can be a problem in cream produced on farms for local sale. Even so he considered that the latter problem could easily be resolved by improvements in hygiene and temperature control. This sanguine view has been challenged by a recent survey of creams in the FGR. Schaal (1984) investigated 3449 samples over a 5-year period; about half of the samples were examined immediately on receipt and the others at the sell-by date with storage at temperatures recommended by the manufacturers. Of all the spoilage samples, 4.4% manifested yeast or mould growth. Whipped cream (sucrose-containing) tended to be spoiled by bacteria. In contrast, yeasts (unnamed) were the causative agents in 48% of the samples of sour cream deemed to be spoilt on receipt and of 74% of those spoilt at the sell-by date. A sensory panel found samples containing>10^5 yeasts ml^{-1} to be unsatisfactory because of yeast odours or flavours. Ottaviani and Disegna (1987) state that Rhodotorula mucilaginosa may occasionally cause a bitter taste and "tiny coagulation flocks" in pasteurized cream.

B Sweetened condensed milk

Sweetened condensed milk, an intermediate moisture product (a_w 0.83), is intended for long term storage at ambient temperature. During manufacture milk is stored at 82-100°C for 10-30 min and a 65% (w/v) sucrose syrup added before water removal by vacuum evaporation (49-57°C). The milk lactose is crystallised (the condensed milk is seeded with lactose crystals) during vigorous agitation for upwards of 1 hr. The finished product is then packed

in cans (sterilized by gas jets) for domestic or drums for commercial use (Foster et al., 1958). It is evident from this generalised account of production methods that there are many opportunities for both airborne and equipment-mediated contamination of sweetened condensed milk unless GMP is practised. Walker and Ayres (1970) noted that spoilage, indicated by foaming of the product and distortion of the container, was commonly caused by Candida globosa and Candida lactis-condensi. Both are tolerant of low a_w and both ferment sucrose with gas production. It will be recalled (pp.32) that both yeasts are common contaminants of sugar, syrups and intermediary refinery samples (Scarr and Rose, 1966). It is tempting to speculate, therefore, that they are well adapted to grow and/or survive in environments and on equipment soiled with sugar residues.

C Cheese

Early microbiological investigations (Walker and Ayres, 1970) of cottage cheese, one of the simplest forms of cheese, demonstrated yeast involvement in spoilage. Yeast growth resulted in discoloration of the surface; fermentation of lactose was associated with a yeasty-odour. Loss of aroma was caused by the breakdown of acetylmethylcarbinol. The last mentioned also occurs when buttermilk is contaminated with yeasts (Wang and Frank, 1981). According to Marth (1987) Rhodotorula rubra and Candida famata are the commonest causes of discoloration. The former initially produces vivid pink spots on the surface of cheese which is eventually covered with a pink slime. Candida famata, a very common

contaminant of dairy products (Table VIII), produces a yellow slime. Marth was of the opinion that cottage cheese ought to be freed of yeast contaminants by the two heat processes which it receives, the initial pasteurization of the milk and the cooking of the curd (120-140^{o}F for 1½-2hr). He concluded that soiled equipment, aerosols and, in particular, the water used to wash the curd were the principal sources of contamination. He also commented that the addition of contaminated vegetables to creamed cheese could well negate procedures designed for the production of a commodity with low levels of yeast contamination.

Upwards of 50% of the annual production of cottage cheese in the UK is now supplemented with vegetables, fruit, meat and fish. Brocklehurst and Lund (1985) investigated changes in the numbers and types of yeasts in cottage cheese varieties (28 batches) obtained from three manufacturers during storage at 7^{o}C. The levels of contamination of products from retailers were determined by the sell-by date. Yeasts belonging to the following genera were isolated (in parenthesis, number of batches of products in which members of a genus were found): Candida (15), Cryptococcus (10), Pichia (2), Sporoblomyces (1); Candida lipolytica (7) and Cryptococcus (5) were the commonest isolates and Pichia onychis was the only fermentative isolate. Of the genera listed above, only Pichia and Sporobolomyces are not of common occurrence in dairy products (Table VIII). It is also noteworthy that only one fermentative yeast was isolated from supplemented cottage cheese, whereas such organisms dominate the yeast flora of yoghurts supplemented with non-dairy ingredients (Table IX). The levels of

Table IX The occurrence and properties of yeasts associated with yoghurts

Genus Species	Yeast associated with yoghurts		Properties of yeast isolated from yoghurts*					Growth (°C)**			
	Spoiled	Normal	Glucose	Galactose	Lactose	Sucrose	Lactate	5°C	10°C	37°C	44°C
Brettanomyces intermedius	+*	+**	O	O	-	Fo	A	-	+	O	O
Candida famata	+	+	O	O	-	Fo	A	+	+	O	O
guilliermondii	+	-	Fo	FA	-	FA	A				
holmii	-	+	FA	-	-	-	A	-	+	+	-
krusei	-	+	FA	-	-	-	A	O	+	+	-
lusitaniae	+	-	Fo	-A	-	-A	-	O	O	O	O
parapsilosis	-	+	-A	-A	-	-	O	O	+	+	-
rugosa	-	+									
versatilis	+	-	Fo	-	-	-	+	O	O	O	O
Kloeckera apiculata	+	+	FA	FA	FA	FA	A	+ -	+	+	+ -
Kluyveromyces marxianus	+	-	Fo	FA	Fa	Fa	A	O	O	O	O
Pichia burtonii	-	+	O	O	-	Fo	-	-	+	O	O
fermentans	+	-	Fo	-	-	-	+				
toletana	-	+	O	O	-	-	A	-	+	O	O
Rhodotorula rubra	+	+	O	O	-	-	A	+	+	O	O
Saccharomyces cerevisiae	+	+	FA	FA	-	FA	A	+ -	+	+	-
Torulaspora delbrueckii	+	-	Fo	-	-	FA	A	O	O	O	O
Zygosaccharomyces microellipsoides	+	-	Fo	FA	-	FA	A	O	O	O	O

* 1. Spillmann and Geiges (1983) - Yeasts isolated from 31 "blown" yoghurts - 25 with and 6 without fruit.

** 2. Taken from Suriyarachchi and Fleet (1981), Green and Ibe (1987) and Comi et al. (1982).

\+ Based on Suriyarachchi and Fleet (1981), Green and Ibe (1987) and Spillman and Geiges (1983):

a: not tested, Growth good (+), weak (+), absent (-), fermentation/assimilation (F/A), assimilation (A), weak fermentation (Fo). "According to Rašić and Kurmann(1978), some of the isolations of Sousa and Uden (1958) grew in yoghurt at 3°C.

yeast contamination in creamed cheese varieties (50 batches; pH range, 4.5-5.1) obtained from retailers and examined at the sell-by date ranged from (log_{10}) > 2.0 (38 batches) to 6.1 g^{-1}. With samples taken directly from manufacturers (28 batches), the initial yeast counts (log_{10}) in 25 batches were < $2.0g^{-1}$. By the last day of the intended storage life at 7°C, yeast growth had occurred in 16 of the 28 batches, the median count at this time being (log_{10}) 3.6 g^{-1} (range, 3.0 - 5.6 g^{-1}). The following yeasts grew in some but not all of the varieties of cheeses included in this study: Candida famata, C. lipolytica, C. sake, C. zeylanoides, Cryptococcus laurentii, Cryp. albidus, Cryp. infirmo-miniatus and Sporobolomyces roseus. In general growth occurred from the 4th day of storage onwards but rarely did the numbers of yeasts equal those of bacteria. Indeed yeasts were associated with spoilage on only one occasion, Sporobolomyces roseus formed red colonies on the surface of unsupplemented cottage cheese. Brocklehurst and Lund were of the opinion that the sources of contamination noted by Marth (1987) probably contributed yeasts to the products included in their study. In practice, of course, their observations do suggest that such contamination does not normally pose problems providing GMP is linked with critical control of chill storage.

"Unsanitary practices" on the farm or in the dairy (Walker and Ayres, 1970) can contaminate hard cheeses with lactose-fermenting yeasts such as Candida kefyr. Growth of these yeasts with "gassy fermentation" may lead to the development of off-flavour and gas bubbles in Cheddar and Swiss-type cheese. A recent study (Spolaor et al., 1987) of Italian soft cheeses revealed that the following

were common contaminants of the atmosphere in a factory, on working surfaces, in curing brine and/or on the cheese surface: Candida humicola, kluyveromyces marxianus var. marxianus, Kluyveromyces marxianus var. lactis, Candida krusei, Debaryomyces hansenii and Rhodotorula rubra. Candida humicola was the most common contaminant in all sites and, as would be expected, Deb. hansenii was associated with the brine. Other studies have shown that the former species occurred on the hands of workers in a milk processing factory (Caramello et al., 1987). In a study of ricotta cheese, Ottogalli et al. (1981) noted that the yeasts which contaminated heat-treated whey were also present in the atmosphere and on the hands of the cheese workers. Debaryomyces hansenii, strains of Kluyveromyces marxianus and to a lesser extent Pichia spp. and Saccharomyces cerevisiae (Table VIII) are commonly isolated from Roquefort, Cabrales, Saint Nectaire, Cantal and Camembert (Choisy et al., 1986). These yeast probably contribute to the organoleptic properties of such cheeses providing growth is not excessive and that it occurs at an appropriate stage in processing. Thus CO_2 arising from lactose fermentation by yeast in the first few days of ripening of Roquefort results in small holes being formed in the curd. According to Choisy et al. (1986) "these may be useful..., they assist the implantation of Penicillium roqueforti". Excessive gas production exaggerates hole formation - "early swelling" (Ottaviani and Disegna, 1987) - and is considered a manufacturing fault. Spoilage need not result from yeasts in or on cheese stored under normal conditions. If, however, such cheese is used in the preparation of food, then yeast spoilage may occur. With pizzas stored at chill temperatures, yeast growth in the cheese topping may

well cause spoilage (Seiler, 1980).

D Fermented milks

A diverse range of fermented milks has evolved in various cultures in Europe, the Middle East, and Africa. Mesophilic and thermophilic lactic acid bacteria are primarily responsible for lactose fermentation and the production of a variety of desirable flavours (Marshall, 1987). Yeasts also contribute to the organoleptic properties of a wide range of products (Iwasawa et al., 1982; Baroudi and Collins, 1975; Kontusaari et al; 1985; Saxelin et al., 1986). In others they cause the loss of desirable attributes. Thus yeast (unnamed) growth in buttermilk leads to loss of flavour and aroma as a consequence of the breakdown of acetylmethylcarbinol (Wang and Frank, 1981).

According to Rašić and Kurmann (1978) there was considerable debate among early investigators about the role of yeasts in yoghurt fermentation. These authors concluded that yeasts are merely chance contaminants of natural yoghurts. Soulides (1956) appears to have been one of the first workers to associate yeast contamination (the lactose - fermenting Candida kefyr) with the spoilage of natural yoghurt - "Contamination with yeasts resulted in a gassy-alcoholic fermentation and a fruity odour...." Judging from the literature yeast spoilage was uncommon before the introduction of yoghurts supplemented with fruit, nuts, sugar and flavouring agents. Shortly after the widespread marketing of this new range of products, Tilbury et al. (1974) reported the isolation in the UK of Candida

famata, C. versatilis, C. pelliculosa, C. intermedia and Hansenula anomala. According to Davis (1971) yoghurts ought not to contain more than 100 yeast cells ml^{-1}, and if properly stored at 5°C, a shelf-life of 3-4 weeks is to be expected. The cardinal importance of minimizing yeast contamination in order to ensure the shelf life of yoghurt was stressed by Ottaviani and Disegna (1987). In a review of yeasts in dairy products, these authors identified two important sources of contamination. In exceptional circumstances yoghurts are infected from depots within the processing plant. Kluyveromyces marxianus var. marxianus is invariably present in such depots. Fruit syrups are the major source of infection. They noted that the low a_w of such syrups may well inhibit some of the yeasts which they harbour but that such stasis is removed by diluting the syrup with yoghurt.

Recent surveys suggest that this low level of contamination is not always achieved. Varabioff (1983), who examined 100 samples of many types of yoghurts from wholesale distributors in Brisbane, found that 72% contained from $10-10^4$ yeasts g^{-1} on arrival in the laboratory. With storage at 7°C until the sell-by date, 24% had counts of 10^4 g^{-1} or more. Suriyarachchi and Fleet (1981) found that 45% of 128 yoghurts obtained from retailers in Sydney contained more than 10^3 yeasts g^{-1}. In another survey of yoghurts from retail outlets in this city (40 samples), Fleet and Mian (1987) found that only 2% had counts less than 10^3 g^{-1}. Similar levels of yeast contamination were obtained in yoghurts purchased from supermarkets and hawkers in Lagos (Green and Ibe, 1987). The lowest counts were obtained with yoghurts supplemented with strawberry fluid and the

highest with stirred natural yoghurts. A survey of plain yoghurts obtained from factories in Saudi Arabia revealed very low levels of yeast contamination at the time of production (Salji et al., 1987). Limited growth occurred when the yoghurts were stored at 7°C and extensive growth with storage at 10°C or 15°C for 14 days. Yeast growth in plain and fruit supplemented yoghurts has been demonstrated with storage at 5°C (Fleet and Mian, 1987) at 10°C but not at 0°C by Green and Ibe (1987). According to Rašić and Kurmann (1975), Sousa and Uden (1958) found that yeasts multiplied in yoghurt at 3°C. These observations demonstrate that chill storage cannot be relied upon to prevent yeast growth in this commodity. Representatives of the six genera of yeasts isolated in the surveys discussed above fermented the sugars, galactose and glucose, arising from lactose hydrolysis and sucrose, a commonly used sweetening agent in yoghurts (Table IX). Ottaviana and Disegna (1987) refer to studies in which gas production was caused by Candida parapsilosis growing synergistically with Lactobacillus bulgaricus, the latter providing a fermentable substrate for the former. Many isolates assimilated lactate, the major product of lactose fermentation by the starter cultures.

As was noted in the Introduction, novel environments favouring yeast growth may result form modifications of traditional foods to meet the ever- changing demands of the consumer. The discussions above of cottage cheese and yoghurt have revealed that such modifications can result in spoilage. Frequent references in the literature to yeasts' tolerance of sorbate or propionate in yoghurts (eg. Comi et al., 1982; Suriyarachchi and Fleet, 1981) and cottage

cheese (Brocklehurst and Lund, 1985), suggest that GMP alone cannot be relied upon to curtail yeast growth should contamination occur.

E Butter and margarine

The commodities included in this section consist of two phases, a continuous fat and a discontinuous aqueous phase. Microbial growth is almost invariably confined to the latter. According to Mossel (1970b) the following two factors play a role in the enrichment and the extent of growth of micro-organisms. The composition of the liquid in respect of pH, nutrients, concentration of NaCl and presence/absence of antimicrobial factors such as sorbate or propionate and, the extent of dispersal of the liquid phase. Microbial growth is curtailed if the water occurs in a large number of very small droplets distributed evenly through the fat phase. During the manufacture of margarine, shear forces disrupt the aqueous phase (16% of the whole formulation) such that monoglyceride-coated water droplets of ca. 3 μm diam. are produced. One gram of finished product contains ca. 10^{10} such droplets (Anon, 1988a). Bullock and Kenney (1969) investigated the contribution of the size of the water droplets on the microbiological stability of a water-in-fat type emulsion (a "low calorie" spread) which was intended to be a substitute for butter. When the majority of droplets were in the size range 3-20 μm, there was only a 1-log cycle increase in the size of the bacterial population during 4 weeks storage at 7.2°C. When many of the droplets exceeded 50 μm diam, the counts increased by 3-4 log cycles in the same storage period. The microbiological stability of these types of products

is also partly dependent upon the maintenance of droplet integrity.

From their review of the literature, Walker and Ayres (1970) concluded that: (1) the presence of yeast in butter was indicative of mistreatment of cream, use of unclean equipment, lack of adequate refrigeration or improper pasteurization, and (2) "the importance of yeast in the deterioration of butter has not been conclusively established". According to Ottaviani and Disegna (1987) lipolytic yeasts (unnamed) may cause rancidity of butter and pigmented ones produce coloured slime on the surface. They identified many sources of contamination of butter, raw milk, non-pasteurised cream, wooden churns, packaging machines, wrapping materials and workers' hands.

In his comprehensive review of microbiology of margarines, Mossel(1970b) assumed that most would have an aqueous phase of pH $\leq$ 4.9, a NaCl content of ca. 6% and a very low content of nutrients. The last mentioned would allow the growth of yeasts and moulds rather than that of the more nutritionally demanding lactic acid bacteria. He was of the opinion that a 1-log cycle increase in the numbers of non-lipolytic yeasts (unnamed) in a margarine "is no cause for alarm". Counts of 10^6 g^{-1} lead to the development of yeasty off-flavours. An entirely different situation obtains when contamination occurs with lipolytic yeasts which cause ketone rancidity or soapy off-tastes due to simple hydrolysis of glycerides. Castañon and Iñigo (1973) claim that the lipolytic Candida lipolytica, a common contaminant of spoiled margarines (Castañon & Iñigo, 1971), is of particular importance in the spoilage process. Through the breakdown of fat, this yeast changes

the stability of the emulsion and initiates a sucession of microorganisms such that spoilage is accentuated. Indeed they claimed that other contaminants will not grow if C. lipolytica fails to grow.

V CEREAL - BASED PRODUCTS

The term cereal-based rather than bakery products was chosen as the title for this section because of the need to direct attention at yeast spoilage of doughs, pastries and products such as cakes (Seiler, 1978). The alternative title would encompass a vast array of products and complex ecosystems. Some of these have been considered elsewhere in this Chapter, for example spoilage of marzipan (pp. 34), fondant coatings on buns (Table VI) and jam (pp. 34). Innovation in the bakery industry, especially in the production of oven-ready savoury products, has produced commodities offering a diverse range of ecosystems for microbial growth. With pizzas, for example, the cheese, olives etc. in toppings may on occasions support yeast growth during chill storage (Seiler, 1980). The migration of tomato juice to the dough- filling interface may support growth of both yeasts and lactic acid bacteria. The latter are associated with fermentation and resultant alcoholic off-odours and distortion of a pack as a result of gas production.

Theoretically an infection route linking the arable field and bake-house can be defined viz cereals in the field -> grain stores (an unstable, man-made ecosystems according to Lacey 1988a,b) -> flour mills and silos -> bake-house. The study of 178 different

samples of oats, wheat, barley, corn and rye by Richter and Thalmann (1983) resulted in the identification of the following: Candida, Cryptococcus, Hansenula, Kloeckera, Pichia, Rhodotorula, Saccharomyces and Trichosporon. Representatives of these genera have been mentioned on many occasions in this Chapter (see Appendix). The contamination and subsequent storage-life of various types of doughs and breads have been critically reviewed by Spicher (1986b). According to Seiler (1980, 1982) bread with undesirable odours and flavours may occur when yeasts (unnamed) other than bakers' yeast are present in dough. He noted that this uncommon problem was often associated with the use of poor quality ingredients, the build up of 'foreign yeasts' on equipment and long fermentation periods for doughs. The main risk areas for the contamination of confectionery products and pertinent control measures have been discussed by Ulrich (1981). Yeast problems in pastry can be exacerbated by the re-use of trimmings. One of us (RGB) has on several occasions investigated yeasty off-flavours in the raw pastry of meat pies intended for deep-frozen storage. In all cases excessive re-use, together with inadequate stock control of trimmings and contamination of pastry with meat juices, contributed to a problem which tends to occur mainly in the summer months. As noted by Seiler (1982), adequate plant hygiene is essential in order to minimize contamination of dough and pastries with yeasts. With spiced doughs for bun production, unusual substrates in the condiments may well be metabolized by yeasts with the production of off-flavours.

Seiler (1980) stressed the importance of baking in the control

of yeast contamination - and hence spoilage problems - of bread. It needs to be stressed that the biological stability of a loaf is compromised by the modern practice of slicing it for the convenience of the consumer. The crust on a loaf forms a relatively effective barrier to yeast spoilage because of its low a_w (0.30-0.50). The contents have a much higher a_w (0.96-0.98) and, if seeded with yeasts by unclean knives, spoilage will be an inevitable outcome of extended storage in humid environments at ambient temperatures (Seiler, 1988). Indeed, spoilage due to yeast growth commonly becomes a problem when plant hygiene is inadequate. The build up of damp debris on racks, conveyor belts and slicing machines is a common feature of poor housekeeping. As many of the causative organisms of spoilage of sliced bread are in the raw dough, workers who prepare the latter ought not to have contact with the cooked product. It is evident from the summary of recent investigations that a diverse range of yeasts have been associated with three distinct forms of spoilage; off-odours, mould growth and chalky white "mould" (Table X).

Rapid growth of yeasts and moulds (unnamed) occurs with ambient storage of tortillas, a Mexican pastry produced from corn flour (Tellez-Giron et al., 1988). According to these authors the "most popular and economical" method of extending shelf-life, the addition of hydrated lime to give a pH of > 9.0, causes an unacceptable yellowing of the product. They extended shelf-life at ambient temperature by the addition of propionate or sorbate to tortillas acidified with fumaric acid.

Table X Yeasts associated with spoilages of bread[+]

Category product	Manifestation of spoilage	Yeasts involved	References
Bread[+]	Off-odour	A range of yeasts have been suspected of causing this problem; *Saccharomyces cerevisiae* is most commonly encountered.	Seiler (1980)
	Surface growth	*Saccharomyces cerevisiae*, *Candida humicola* *Zygosaccharomyces bailii* *Saccharomycopsis fibuligera* *Pichia burtonii*	Spicher (1985)
	Chalky White "mould"	*Saccharomycopsis figuligera* *Zygosaccharomyces bailii*, *Pichia burtonii*	Spicher (1984)
		Saccharomyces cerevisiae *Candida parapsilosis* *Torulaspora delbrueckii* *Candida humicola* *Pichia membranaefaciens*	Spicher (1986a)
		Pichia burtonii	Ponte & Tsen (1987)
		Hansenula anomala var. *anomala*	Seiler (1980)
		Geotrichum	
Rye-bread	Not specified	*Saccharomyces cerevisiae* *Rhodotorula rubra* *Saccharomycopsis fibuligera* *Debaryomyces hansenii*	Hartog and Kuik (1984)

+ For specific reviews, see Seiler (1980) and Spicher (1986b)

VI SAUCES AND SALADS

This section deals mainly with acid products that may suffer spoilage because of poor manufacturing practices or contamination during use (Table XI).

A. Mayonnaise and salad dressings

Mayonnaise is a semi-solid emulsion (pH 3.6-4.0) containing about 0.29-0.5% acetic acid. The aqueous phase contains (%), salt (9-11), and sugar (7-10). A salad dressing (pH 3.2-3.9) is essentially a mayonnaise supplemented with a cooked starch-paste. The aqueous phase contains, salt (3-4%) and sugar (20-30%). These two commodities will therefore enrich organisms tolerant of both a slightly diminished a_w and undissociated acetic acid. Approximately 0.5% of the undissocated form of this acid inhibits yeast growth in laboratory media (Baird-Parker, 1986). Studies reviewed by Smittle (1977) demonstrated the occurrence of yeasts, lactobacilli and Bacillus spp. in spoiled mayonnaise and salad dressings. Kurtzman et al. (1971), who surveyed brands of spoiled mayonnaise and salad dressings manufactured throughout the USA, isolated Zygosaccharomyces bailii from two-thirds and Lactobacillus fructivorans from one-third of the samples. In their review of the older literature, Walker and Ayres (1970) concluded that contaminated processing equipment was usually responsible for spoilage (gas production). Dennis and Buhagiar (1980) considered vegetable processing equipment to be an important source of contamination of mayonnaise-based salads. Kurtzman et al. (1971)

Table XI Yeasts associated with the spoilage of dressings, sauces and salads

Commodity	Manifestation of spoilage	Yeasts involved	References
Salad dressing*	Gas formation	*Zygosaccharomyces bailii* *Torulaspora delbrueckii*	Kurtzman et al. (1971),
Mayonnaise*	Gas formation	*Zygosaccharomyces bailii*	Kurtzman et al. (1971), Smittle (1977).
Salads			
coleslaw	Off-odours; gas formation	*Saccharomyces exiguus* *Zygosaccharomyces rouxii*	Dennis (1983), Dennis and Buhagiar (1980).
potato"	Gas formation	*Saccharomyces exiguus*, *Candida sake*, *C. lambica*, *C. lipolytica*	Kirsop and Brocklehurst (1982), Brocklehurst and Lund (1984).
vegetable	Gas formation Film formation	*Saccharomyces exiguus* *Pichia membranaefaciens*	Brocklehurst and Lund (1984).
florida	Gas formation	*Saccharomyces exiguus*	
mixed vegetable and fruit	Gas formation; off-odour	*Saccharomyces* spp.	Dennis (1983).

Table XI continued

Commodity	Manifestation of spoilage	Yeasts involved	References
Tomato sauce	Not stated Film formation	*Candida krusei* *Pichia membranaefaciens*	Pitt and Hocking (1985).
Tomato ketchup	Not stated	*Zygosaccharomyces bailii*	Mori et al. (1971).
Raw soy sauce	Gas formation White dry pellicles	*Zygosaccharomyces* spp. *Pichia* spp.	Onishi (1963).

* Products obtained from Japanese retailers were surveyed by Udagawa et al. (1981); "Japanese chilled potato and macaroni salads were investigated by Kobatake and Kurata (1980a).

noted that the production of gas may not become evident until sometime after a contaminated product has left a factory. They surmised that this was due to a "delayed" fermentation of sucrose by Zygosaccharomyces spp.

B Mayonnaise-based salads

There has been a marked increase in the production of mayonnaise-based salads in recent years. Such products have a shelf-life of 6-14 days with storage at 4-6°C (Tunaley and Brocklehurst, 1982). A longer shelf-life is achieved in many European countries through the use of preservatives (Kirsop and Brocklehurst, 1982). Complex physico-chemical changes occur during the storage of these multiphase systems, for example in coleslaw, cabbage, onions and carrots are suspended in an oil-in-water emulsion. Following preparation there is a substantial transfer of water from vegetable tissue to the mayonnaise. Within 6 hours of manufacture, a considerable proportion of the acetic acid has diffused in the opposite direction. Consequently there is a change in the pH (ca. 3.5 - 4.5) of the mayonnaise and a resulting diminution in the concentration of undissociated acetic acid (Dennis, 1983; Dennis and Buhagiar, 1980; Tunaley and Brocklehurst, 1982; Kirsop and Brocklehurst, 1982; Brocklehurst and Lund, 1985). Brocklehurst et al. (1983) studied mayonnaise, with or without vegetable supplements, which had been inoculated with Zygosaccharomyces rouxii and Saccharomyces exiguus. Both yeasts have been associated with the spoilage of coleslaw (Kirsop and Brocklehurst, 1982). The yeasts were killed in mayonnaise alone,

death resulting from the action of acetic acid and to a lesser extent NaCl. The rate of death was accelerated by a rise in temperature. Yeasts grew in mayonnaise supplemented with cabbage and carrot as a consequence of the diminished concentration of acetic acid *per se* and of the undissociated acid in particular, the latter being caused by an alkaline drift of one pH unit in the mayonnaise. Growth was inhibited by the addition of chopped onions to the mixture. Volatile materials in the onions were tentatively identified as the cause of yeast death. Electron optic studies showed that yeasts grew in the aqueous phase of the mayonnaise but only to a limited extent at the interphase of vegetables and emulsion and within plant tissue (Dennis, 1983).

A detailed study of a range of mayonnaise-based salads (Brocklehurst and Lund, 1984) underlined the importance of undissociated acetic acid in the enrichment of micro-organisms, particularly that of yeast at the expense of lactic acid bacteria. Spoilage did not occur in Spanish salad (pH 3.7; concentration of acetic acid, 0.6% and undissociated acid, 0.55%). Gas formation was observed in the mayonnaise of Florida salad (pineapples and oranges included with vegetables) after 13-days at 10°C. In this instance the mayonnaise was pH 3.5 and contained 0.5% acetic acid and 0.48% undissociated acetic acid. With storage at 10°C, large populations of *Sacch. exiguus*, *Pichia membranaefaciens* and lactobacilli were formed in a vegetable salad (pH 4.0; 0.5%; 0.45%). Gas formation was noted after 8 days and was associated with the growth of *Sacch. exiguus*, *P. membranaefaciens* formed colonies on the surface of the salad. No growth occurred with storage at 5°C. Gas formation was

detected after 21 days at 10°C with a potato salad (pH 4.4; 0.1%; 0.07%) stored at 10°C, lactobacilli dominated the spoilage flora, with a climax population of $1x10^9$ organisms g^{-1}. Populations of $10^{6-7}g^{-1}$ were attained by Sacch. exiguus, Candida sake, Candida lambica and Geotrichum candidum. Candida lipolytica formed a population of less than 10^6g^{-1}. Candida zeylanoides was the dominant organism (ca 10^6g^- at 28 days) in potato salad stored at 5°C; C. sake, C. lambica and C. lipolytica formed populations of ca 10^{5-6} after 24-28 days storage. Although bacteria also grew extensively in this type of salad, spoilage was associated with the growth of G. candidum. This study also raised questions about the interpretation of gas production in mayonnaise-based salads; the older literature tends to equate gas production with spoilage by yeasts (Walker and Ayres, 1970). It was obviously an indicator of yeast spoilage in a Spanish salad. With the vegetable salad, yeasts as well as lactobacilli may well have produced gas. Indeed it needs to be stressed that homolactic lactobacilli produce CO_2 through the decarboxylation of malic and citric acid thereby contributing to "bloater" formation in cucumber (Fleming et al., 1973a,b).

C Sauces

Walker and Ayres (1970) noted that yeasts were the spoilage organisms of tomato sauce. Mori et al. (1971) indentified the spoilage organism in Japanese ketchup with Zygosacch. bailii. From a limited study of an unnamed yeast from spoiled ketchup, Sorrells and Leonard (1988) concluded that tolerance to acetic acid "involves an energy-requiring system". According to Flores et al. (1988) acid

(unspecified) and low pH (4.1-4.5) are the sole preservative systems in restaurant-type Mexican hot sauces which do not normally receive any heat treatment during production. Indeed spoilage can occur within 3-5 days of production of sauce stored at ambient temperature. They noted a 4-log cycle increase in the number of yeasts (unnamed) in an inoculated sauce during 10-days storage at 25°C.

VII MEAT, POULTRY AND OTHER PROTEINACEOUS FOODS

Yeasts are rarely implicated in the spoilage of red meat and poultry (Walker and Ayres, 1970). They have been associated with the spoilage of a range of cured meats (Table XII). This section focusses attention on processing and storage methods which modify the unstable ecosystems of meat and meat products such that yeasts compete more effectively with bacteria.

A Occurence

A review of 26 studies (conducted between 1919-1984) revealed that representatives of 14 genera of yeasts have been isolated from red meats, sausages and poultry (Dillon, 1988). The following genera were most commonly isolated (in parenthesis, number of studies in which members of a genus were isolated): Candida (21), Debaryomyces (14), Rhodotorula (13), Trichosporon (11), Cryptococcus (7), Pichia (4) and Hansenula (4). Cryptococcus, Candida and Rhodotorula were the principal yeast contaminants of a sheep slaughter house investigated by Baxter and Illston (1976, 1977).

Table XII Factors that are conducive to yeast growth on meat and meat products*

Factor Commodity	Yeasts isolated	References
Storage at -5°C		
Lamb	*Cryptococcus laurentii* var. *laurentii*	Lowry (1984).
	Cryptococcus infirmo-miniatus	Lowry and Gill (1984).
	Trichosporon pullulans	Winger and Lowry (1983).
	Candida zeylanoides	
Nutrient limitation		
Animal Fat	Unnamed	Lea (1931).
Sulphite		
British fresh sausages	*Debaryomyces hansenii*	Dalton et al. (1984).
	Candida zeylanoides	Banks and Board (1987).
	Pichia membranaefaciens	Banks et al. (1985b).
Minced lamb	*Candida famata*	Dillon (1988).
	Candida humicola	
	Candida inconspicua	
	Candida lipolytica	
	Candida mesenterica	
	Candida norvegica	
	Candida sake	
	Candida vini	

Table XII continued

Factor Commodity	Yeasts isolated	References
Minced lamb	*Cryptococcus albidus* var. *albidus* *Cryptococcus infirmo-miniatus* *Cryptococcus laurentii* *Rhodotorula minuta* *Rhodotorula rubra* *Trichosporon cutaneum*	
Sulphite/pasteurization		
Skinless sausage	*Candida zeylanoides* *Debaryomyces hansenii* *Cryptococcus* spp. *Rhodotorula* spp. *Candida lipolytica* *Candida* spp.	Tudor and Board (1989a).
Low a_w		
Cured fermented sausages+	*Candida* spp. *Debaryomyces* spp.	Leistner and Bem (1970). Comi and Cantoni (1985).
Cured raw meat	*Debaryomyces hansenii* *Candida famata* unnamed	Smith and Hadlock (1976). Cantoni et al. (1969).

Table XII continued

Factor Commodity	Yeasts isolated	References
Antibiotics		
Poultry	*Rhodotorula* *Cryptococcus* spp. *Saccharomyces* spp.	Ayres et al. (1956). Njoku-Obi et al. (1957). Wells and Stadelman (1958). Walker and Ayres (1959).
Irradiation		
Frankfurters	*Debaryomyces* spp. *Candida famata*	Drake et al. (1959).
Minced beef (with storage at 4°C)	*Candida famata* *Candida lipolytica* *Candida parapsilosis* *Candida sake* *Candida zeylanoides* *Cryptococcus albidus* var. *albidus* *Cryptococcus laurentii* var. *laurentii*	Johannsen et al. (1984).

Table XII continued

Factor Commodity	Yeasts isolated	References
Minced beef contd.	*Trichosporon cutaneum* *Trichosporon pullulans* *Rhodotorula minuta* var. *taxensis* *Rhodotorula rubra*	
Snail meat	*Candida famata*	Nwachukwu and Akpata (1987).

* An extensive list of yeasts associated with a diverse range of meat and meat products was published by Jay (1979). The results of a comprehensive study of the isolation methods for and classification of meat yeasts was published by Hessel-Schmalfuss et al. (1976).

+ Tiwari and Kadis (1981) recorded upwards of a 10-fold increase in the yast counts of the following in 24 h storage at 22°C of samples purchased from delicatessen sections of shops: sausage, pepperoni, Italian salami, cervelat, corned beef, frankfurter, garlic sausage, smoked ham and knackwurst.

Members of the first 3 genera as well as Trichosporon spp. had an unusual distribution in an abattoir studied by Thompson et al, (1985). The yeasts were the dominant contaminants on surfaces above the carcasses, fans refrigeration units and ceilings. Bacteria, particularly coryneforms, were the main ones on the floor and at the bottom of concrete walls. Debaryomyces hansenii, a common contaminant of meat processing factories (Walker and Ayres 1970), is particularly abundant in and around tanks of brine used for meat curing (Costilow et al., 1954). Dalton et al. (1984) noted that the yeasts of British fresh sausages (Table XII) also occurred on equipment throughout a sausage factory. Dillon (1988) traced yeast contamination of minced lamb back through an abattoir to pastures where sheep were being fattened for slaughter. Poultry support a yeast flora which reflects that on contaminated processing equipment. In early stages this is dominated by Trichosporon and Cryptococcus spp, in the later stages by non-filamentous yeasts including Candida and Debaryomyces spp. (Tudor and Board, unpublished observations).

Although the yeast : bacteria ratio was highly variable in the 111 samples of ground beef investigated by Jay and Margitic (1981), there was a trend for high yeast counts to be associated with heavy bacterial infection. Rhodotorula, Trichosporon, Cryptococcus and Candida spp. were the main yeast contaminants of American (Hsieh and Jay, 1984) and Italian (Comi and Cantoni, 1985) minced beef. Candida spp. were the dominant yeast contaminants of the samples, especially spoiled ones, included in the study by Hsieh and Jay. Nychas (1984) also found this genus to be the main contaminant of

minced beef obtained from supermarkets and butchers' shops in the UK. Chill storage of Italian minced beef was associated with the growth of Trichosporon spp. (Comi and Cantoni, 1985). Candida and Trichosporon spp. were isolated from the slime developing on meat during chill storage (Comi and Cantoni, 1984). This brief survey has shown that red meat and poultry tend to be contaminated with the representatives of a relatively small number of yeast genera, all of which occur on equipment and fittings in meat and poultry processing plants.

B Spoilage

Traditionally yeast spoilage has been associated with cured meats, fermented sausages etc. (Table XII) in which bacterial growth is prevented by low water activities (a_w 0.83 - 0.87) and, in some cases, acid conditions (Walker and Ayres, 1970). Antibiotic - inhibition of bacteria on poultry also results in extensive yeast growth during chill storage (Table XII). Yeasts are seldom mentioned in the recent literature on microbial spoilage of the chilled meat products that are sold in the supermarkets of developed countries (Nychas et al., 1988). Some studies (eg. Banks and Board, 1987) have shown that yeasts do grow in such products; their growth has not been associated with changes in organoleptic attributes. In practice the yeasts fail to compete effectively with the nutritionally - versatile, rapidly growing bacteria (mainly pseudomonads) on chilled meats exposed to normal atmospheres, or with the lactic acid bacteria and Brochothrix thermosphacta on products stored in CO_2 - enriched atmospheres. Of course, the

ecosystems enriching these two groups of bacteria are very unstable and changes in production methods or storage conditions may well result in the election of yeasts. This was evidently the case with lamb stored at -5°C (Table XII), where low water activity resulting from ice formation in meat tissue was probably the main elective factor. Indeed this factor needs to be considered should an extension of the shelf-life of perishable meat products be sought by "super-chilling" (-1 to -5°C) of items awaiting distribution from depots.

Some studies have shown that the yeast : bacteria ratio in the initial contamination of highly unstable ecosystems in meat products determines the size of the populations formed by these two groups of organisms during product storage, thereby influencing the nature of eventual spoilage. Gamma irradation of minced meat (Johannsen et al., 1984) killed many of the Gram-negative bacteria, the dominant contaminants at the time of mincing, but relatively few yeasts. With storage at 4°C, Candida famata, Candida lipolytica and Candida parapsilosis as well as other yeasts (Table XII) formed large populations. When the numbers of yeasts roughly equalled those of bacteria at the time of manufacture of British fresh sausages containing sulphite (Dowdell and Board 1971; Brown, 1977), the extent of growth of unnamed yeasts was greater than that of lactic acid bacteria and Brochothrix thermosphacta. This resulted in the development of yeasty off-odours and a thick film of yeast on the surface of sausages. A parity in the yeast : bacterial counts is an uncommon feature in freshly manufactured sausages and the underlying factors for this situation were not identified by Dowdell and Board

(1971) and Brown (1977). Mossel (pers. comm.) surmised that yeasts may outgrow bacteria in meat residues on processing equipment as a consequence of a_w being diminished by evaporation. This phenomenon may also have contributed to the unusual yeast : bacteria ratio in the sausages studied by Dowdell and Board (1971) and perhaps to the curious distribution of bacteria and yeasts in the abattoir studied by Thompson et al. (1985).

C Sulphited meats

Sulphite has a pronounced effect on the composition of the microbial associations as well as the yeast contribution to the microbial biomass developing in minced meat (Dillon, 1988) and British style sausages (Banks et al., 1985b). The associations which develop during storage of unpreserved products at 4 - 7°C are dominated by Gram-negative bacteria, particularly pseudomonads (Nychas et al., 1988). Their growth in nutrient broth is inhibited by free sulphite at concentration above 350 $\mu g\ ml^{-1}$ (Banks and Board, 1982a). Lactobacillus spp. and Brochothrix thermosphacta are tolerant of higher concentrations and in sulphited meats their relatively slow growth appears to give yeasts an opportunity of contributing significantly to the size of the microbial associations when colony forming units are used as an index. In terms of biomass, that formed by the yeasts is equal to or greater than that of bacteria in British style sausages (Dalton et al., 1984). Although yeasts make such an important contribution to the contamination of sulphited sausages, Leads (1979), who attempted to correlate the assessments of a trained taste panel with chemical

changes in stored sausages, was unable to identify the extent of yeast contribution to spoilage. Dillon and Board (1989b) found that yeasts were only minor contaminants (0.05 - 2.0%) vis à vis the total viable count of freshly minced lamb from a butcher's shop. Yeast contribution to contamination declined during storage at 5°C. The addition of sulphite (500 $\mu g\ g^{-1}$) to a sample of this mince led to extensive yeast growth such that after 4 days at 5°C the yeast populations were $7.7 \times 10^6\ g^{-1}$ meat and those of bacteria $4.3 \times 10^6\ g^{-1}$. With heavily contaminated mince from a supermarket, the addition of sulphite again favoured yeast growth but the numbers of bacterial contaminants remained dominant. This is yet another example of the influence of yeast : bacteria ratio on the eventual outcome of microbial development in meat products.

The concentration of free sulphite diminishes progressively during the storage of preserved meat and meat products (Banks et al., 1985b). Dalton (1984) correlated sulphite binding with yeast growth and acetaldehyde accumulation ($r = 0.89$, $n = 26$) in British style sausages. She also demonstrated sulphite-induced production of acetaldehyde by representatives of the following: Debaryomyces, Pichia, and Candida. Dillon (1988) showed that a suitable substrate, glucose or fructose, and a pH more alkaline that 5.5 were essential for sulphite-induced production of acetaldehyde by yeasts isolated from preserved minced lamb. The capability of binding sulphite confers only a temporary advantage to yeasts in preserved meat and meat products (Dalton, 1984); only initially is the proportion of sulphite-binding yeasts slightly greater than that of non-binding yeasts (Dillon and Board, 1989a). It needs to be

stressed that sulphite inhibition of Gram-negative bacteria is removed once the concentration of free sulphite falls below threshold levels (Banks and Board, 1982a). When such levels obtain, the pseudomonads and enterobacteria commence growth and form large populations in British style sausages (Banks et al., 1985b) and minced lamb products (Dillon, 1988). Under normal conditions the renewal of growth of Gram-negative bacteria occurs in products that would be deemed to have spoiled by a discerning person.

The evidence discussed so far has focussed attention on the potential rather than on the actual contribution of yeasts to the spoilage of sulphited meat products. With skinless British style sausages, yeasts are the dominant spoilage organisms (Table XII). In the manufacture of this product, sausage meat in cellophane tubes is exposed to 71°C for 2 min 10 sec. The peripheral proteins of the sausage are coagulated such that shape is retained when a sausage is released from its tube. The heat treatment also has a pasteurizing effect; the outer surface of a sausage released from the cellophane tube is lightly contaminated. Tudor and Board (unpublished observations) have found that colonisation of the surface with a succession of yeasts (Candida zeylanoides, Debaryomyces hansenii, Cryptococcus spp., Rhodotorula spp., Candida lipolytica) causes a yellow paste to form. Such pastes are recognized in commerce as the most common manifestation of microbial spoilage of this particular commodity.

D Snail meat

According to Nwachukwu and Akpata (1987) Candida famata is an important spoilage organism of the meat of the giant West African snail (Archachatina marginata Swainson), especially with storage at ambient temperature ($29^{o}C \pm 1$). As this yeast is apparently a normal resident of the mid gut of the snail, poor evisceration methods would cause heavy contamination of the meat.

VIII SEAFOODS

An intriguing feature of the literature on the microbiological quality of seafoods was highlighted by Ward and Baj (1988). More than 3000 marine creatures are important items of commerce in various parts of the world. In spite of the phylogenetic heterogeneity within this group of commodities, the diversity of environments from which they are harvested, the plethora of harvesting, processing and marketing systems, the literature on seafoods would have us believe that Gram-negative bacteria are the commonest spoilage organisms (Hobbs, 1983; Walker and Ayres, 1970). Indeed Kobatake et al. (1988) recently noted that "there have been almost no studies on the spoilage of seafoods by yeasts". Moreover there appears to be only one dissenting voice against the concensus view that spoilage is the result of aerobic metabolism. Jay (1987) asserted that glycogen-rich molluscan shell-fish undergo fermentative spoilage.

A Raw fish

There is general agreement that the composition of the bacterial flora on fish reflects that of the water in which they live (Ward and Baj, 1988). Limited studies of the yeasts of sea water and fish harvested therefrom partially supports this view. Morris (1975) listed the following as the most commonly occurring species in sea water: Candida parapsilosis, Rhodotorula rubra, Candida tropicalis, Debaryomyces hansenii, Cryptococcus albidus, Cryptococcus laurentii, Candida famata and Candida guilliermondii. Representatives of these four genera of yeasts were isolated from some or all of the seafoods examined in the UK (Ross and Morris, 1965), USA (Phaff et al., 1952; Eklund et al., 1965), Japan (Kobatake and Kurata, 1980b, 1983) and Italy (Comi et al., 1987). Comi and his collaborators, who studied a wide range of fish from the mediterranean, have published the most extensive list of yeast genera associated with fish. In addition to those listed by Morris, they isolated representatives of the following: Pichia, Leucosporidium, Trichosporon, Sporobolomyces, Saccharomycopsis and Hansenula. Even so these workers concluded that "yeasts' role in fish deterioration is unimportant with respect to bacteria". In their survey of chilled roe of sea urchin, sliced raw tuna and the foot of the hen clam obtained from retail outlets, Kobatake and Kurata (1980b) obtained yeast counts of 10^{2-7} g^{-1} of roe and 10^{1-4} g^{-1} of the other two commodities. The recent studies by Kobatake et al. (1987, 1988), who used autoclaved homogenates of common mackeral, black tiger shrimp and neon flying squids as test substrates, showed that the following produced odours and/or slimes

similar to those formed by bacteria: Candida lipolytica, Trichosporon pullulans, Trichosporom cutaneum, Cryptococcus laurentii and Candida sake. Yeasts therefore have the potential to cause spoilage should conditions favour their growth at the expense of bacteria. Large yeasts populations are formed during the storage of irradiated fish or that treated with antibacterial agents (Walker and Ayres, 1970).

B Processed Fish

Yeasts appear to be common contaminants of processed fish; occasionally they cause spoilage. Red burong isda, a fermented mixture of raw fish, cooked rice and condiments produced in the central Luzon region of the Philippines, were contaminated with the following (Sakai et al., 1983): Saccharomyces cerevisiae, Candida tropicalis, Candida parapsilosis, Pichia strasburgensis, Zygosaccharomyces rouxii and Pichia carsonii. The last two were isolated from samples having a high salt content. The authors concluded that the yeasts were chance contaminants which probably contributed to subtle differences in the organoleptic properties of different batches of burong isda. Yeast production of fruity off-odours in sugared and salted herring containing nitrate was discussed by Knøchel and Huss (1984). The xerotolerant yeasts responsible for this fault were not identified. Red or pink discoloration of oysters and clams due to the growth of Rhodotorula rubra has been studied over many years (Walker and Ayres, 1970). A recent study by Hood (1983) confirmed many of the early observations. She noted that the majority of freshly harvested

shell-fish harboured Rh. rubra, the highest levels of contamination occurring on fish from water having the highest salinity. There was no appreciable yeast growth during storage of entire shellfish at 8°C; populations on shell-less or fully processed fish increased 100 and 1000-fold respectively. She surmised that extensive washing in the preparation of a fully processed commodity may have leached antimicrobial agents from the fish.

CONCLUDING REMARKS

This extensive review of literature scattered throughout journals dealing mainly with commodities rather than yeast per se has endorsed a precept and identified a concept. It has endorsed the precept discussed by Pitt and Hocking (1985) that yeast spoilage of traditional foods is the outcome of their enrichment by extremes of one or more abiotic factor, low a_w and acid conditions being the main elective factors. Indeed it is such factors that thwart the bacteria, mainly Gram-negative ones having simple nutritional requirements but marked nutritional versatility, that contribute to the microbial associations developing on foods which do not impose extreme conditions of enrichment. The concept relates to the situation which obtains midway between these two extremes. With traditional foods occupying this zone, spoilage is commonly the outcome of extensive growth by acid-tolerant bacteria, lactobacilli or, less commonly, acetic acid bacteria. In the course of this Chapter, many examples of yeast spoilage of such foods have been discussed but in practically all cases, the factor(s) favouring the growth of yeasts over that of bacteria cannot be identified. In a

few instances a yeast-to-bacteria ratio of 1:1 appears to confer an advantage to yeast growth. It needs to be stressed that many of the yeasts (Table XIII) associated with spoilage of foods in this intermediate zone are not in the "top ten" spoilage yeasts listed by Pitt and Hocking (1985) - see Table III. In view of the current developments in food processing, such as the production of a diverse range of ready-to-use salads, dairy desserts etc, there would appear to be an urgent need for research into the interactions between organisms in the second division of spoilage yeasts and acid tolerant spoilage bacteria.

Table XIII Second division yeasts - common food contaminants, opportunist spoilers

Organisms	Examples of spoilage (references)
Candida dattila+	Marzipan, persipan (Windisch and Neumann, 1965).
Candida globosa	Cooked fruit (Moon et al., 1985), Sweetened condensed milk (Walker and Ayres, 1970).
Candida humicola	Bread (Spicher (1986a).
Candida lactis-condensi	Sweetened condensed milk (Walker and Ayres, 1970), sugars, syrups (Scarr and Rose, 1966).
Candida lipolytica+	Cheese (Engel, 1986b), potato salad (Brocklehurst and Lund, 1984), margarines (Castañon and Iñigo, 1971).
Candida parapsilosis	Cooked fruit (Moon et al., 1985).
Candida sake	Potato salad (Brocklehurst and Lund, 1984).
Candida versatilis	Yoghurts (Spillman and Geiges, 1983), chocolate syrup (Tilbury, unpublished).
Candida zeylanoides	Skinless sausage (Tudor and Board, 1989a).
Cryptococcus spp.	Strawberries (Dennis, 1983).
Hansenula anomala+	Bread (Seiler, 1980), syrups (Tilbury, 1976), olives (Vaughn, et al. 1969), mushrooms (Comi et al., 1981).
Hansenula subpelliculosa	Syrup (Bedford, 1942), mango achaar (Van der Reit, 1982).
Kluyveromyces marxianus+	Cooked fruit (Put and de Jong, 1980), yoghurt (Spillman and Geiges, 1983).
Pichia burtonii+	Bread (Ponte and Tsen, 1987).
Pichia fermentans+	Yoghurts (Spillman and Geiges, 1983).
Sporobolomyces roseus	Cottage cheese (Brocklehurst and Lund, 1984).
Torulaspora delbrueckii	Jam (Tilbury, 1976), yoghurts (Spillman and Geiges, 1983).
Trichosporon cutaneum	Kocho (Gashe, 1987), forced rhubarb petioles (Dennis, 1983).
Trichosporon pullulans	Strawberries (Dennis and Buhagiar, 1980).

+ Corresponding perfect/imperfect name given in Appendix B.

CHAPTER 2

IDENTIFICATION OF YEASTS

Methods of screening large numbers of isolates were required, to investigate the ecology of yeasts from meats and poultry and their potential significance as spoilage organisms. Established procedures of classical identification were adapted to accommodate specifically yeasts from the meat environment, thus enabling the rapid recognition of different species and, depending on the objective, further investigations on either identification or behaviour of the isolate.

INTRODUCTION

In order to classify a group of organisms the group must be defined. In the case of the yeasts this is extremely difficult since they may best be described as a category of fungi in which the unicellular state is predominant. The yeast is merely a physical and, in many cases, transient state (Rayner and Boddy, 1988). A system of classification based on the property of unicellularity would be unwise since this would allow many different organisms to be considered as yeasts. Certain environmental conditions allow Mucor spp. to become yeast-like (unicellular) and yet they are not generally regarded as yeasts. Jørgensen (1939) noted that most Mucoraceae produced alcohol from sugar under aerobic and especially under anaerobic conditions and he observed spherical 'yeast-like' cells on the bottom of the liquid. An interesting discussion of the relationships between yeasts and related filamentous fungi is given by Von Arx (1979). The term 'yeasts' needs to be defined more precisely.

The ideal classification is a natural one whereby organisms are grouped according to similarities in habitat, physiology and cell structure. Such criteria are termed phenetic as they reflect affinities based on the phenotype of the organism. The groups (phena) so formed will each contain organisms which have a high overall similarity determined by a large number of features which they all have in common, although not any one in particular. This allows individuals which do not conform in any one way but do so in

many others, to be accommodated in a group. Such an arrangement of organisms permits a logical and informative system of naming and may be used as a key for the classification of unidentified organisms (Sneath, 1978; Sokal, 1985; Austin and Priest, 1986).

A perfect system of classification is one reflecting the evolutionary pathway of organisms (phylogenetic) and has been successfully employed in bacterial taxonomy (Woese, 1987; Schleifer and Kilpper-Balz, 1987). Wickerham (1951) discussed the phylogenetic relationships between species in the genus Hansenula. He was of the opinion that the haploid species represented earlier forms of the genus, some of which had evolved to entirely diploid species. A scheme similar to that used in bacterial taxonomy has not been widely applied to the yeasts since so much information is missing. Recently, advances have been made in terms of species delimitation by using molecular and chemotaxonomic methods and this will be discussed below.

Since, however, the phenotype results from the expression of the genotype then any phenetic system of classification will provide insights into phylogenetic patterns. Indeed, the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics (Wayne, 1988), emphasize that phylogenetically based taxonomic schemes must also show phenotypic consistency. Developments in bacterial taxonomy have progressed faster and further than yeast taxonomy, but one would expect the same concepts to eventually be applied to the yeasts.

There are two means of classifying yeasts, conventional (classical) and non-conventional (ecological). The first of these

stems from Hansen's work on the selection of brewing yeasts in 1888. He laid the basis for the use of phenotypic characters for species delimitation, by differentiating isolates on the basis of cellular and ascosporal morphology, characteristics of growth in liquid media, optimal growth temperatures and their ability to ferment different sugars. As new yeasts have been discovered, subsequent taxonomists have extended Hansen's list of phenotypic characters to include their own. A recent monograph by Barnett et al. (1983) provides a list of 473 yeast species differentiated using 97 tests based on physiological and morphological features. The most comprehensive text to date (Kreger-van Rij, 1984) recognizes about 500 species.

CLASSICAL

The yeast domain contains representatives of the Ascomycota, Basidiomycota and the Deuteromycetes (Fungi Imperfecti). Two groups are recognized: (1) the perfect or teleomorphic yeasts which have a distinct sexual stage illustrated either by the development of an ascus or a basidium, and (2) the imperfect or anamorphic yeasts which fail to express a sexual stage. As can be seen from Table 2.1, of the 500 species described by Kreger-van Rij (1984), 183 are classified as ascomycetes and belong to 33 different genera and 36 are basidiomycetes and belong to 10 genera. Therefore, the first group of yeasts described above contains 219 species but the remaining 281 species have no recognized sexual stage and belong to the deuteromycetes consisting of 17 genera. It has been shown, however, that many of the imperfect yeasts resemble ascomycetes or

Table 2.1 Classification of the yeasts in the Eumycota

Class Subclass Order	Family Subfamily	Genera	No. of Species
Ascomycotina			
Hemiascomycetes			
Endomycetales	Spermophthoraceae	*Coccidiascus*	1
		Metschnikowia	6
		Nematospora	1
	Saccharomycetaceae		
	Schizosaccharomycetoidae	*Schizosaccharomyces*	4
	Nadsonioideae	*Hanseniaspora*	6
		Nadsonia	3
		Saccharomycodes	1
		Wickerhamia	1
	Lipomycetoideae	*Lipomyces*	5
	Saccharomycetoideae	*Ambrosiozyma*	4
		Arthroascus	1
		Citeromyces	1
		Clavispora	1
		Cyniclomyces	1
		Debaryomyces	9
		Dekkera	2
		Guilliermondella	1
		Hansenula	30
		Issatchenkia	4
		Kluyveromyces	11
		Lodderomyces	1
		Pachysolen	1
		Pachytichospora	1
		Pichia	56
		Saccharomyces	7
		Saccharomycopsis	7
		Schwanniomyces	1
		Sporopachydermia	2
		Stephanoascus	1
		Torulaspora	3
		Wickerhamiella	1
		Wingea	1
		Zygosaccharomyces	8
Basidiomycotina			
Ustilaginales	Filobasidiaceae	*Chionosphaera*	1
		Filobasidiella	1
		Filobasidium	3
	Teliospore-forming yeasts	*Leucosporidium*	6
		Rhodosporidium	9
		Sporidiobolus	4
Tremellales	Sirobasidiaceae	*Fibulobasidium*	1
		Sirobasidium	1
	Tremellaceae	*Holtermannia*	1
		Tremella	9
Deuteromycotina			
Blastomycetes	Cryptococcaceae	*Aciculoconidium*	1
		Brettanomyces	9
		Candida	196
		Cryptococcus	19
		Kloeckera	6
		Malassezia	2
		Oosporidium	1
		Phaffia	1
		Rhodotorula	8
		Sarcinosporon	1
		Schisoblastosporion	1
		Sterigmatomyces	6
		Sympodiomyces	1
		Trichosporon	15
		Trigonopsis	1
	Sporobolomycetaceae	*Bullera*	6
		Sporobolomyces	7

Adapted from Kreger-van Rij (1984)

basidiomycetes in terms of features such as cell-wall composition (Weijman, 1979), the ultrastructure of cell-wall and conidiation (Von Arx, 1980) a colour reaction with certain diazonium dyes (Van der Walt and Hopsu-Havu, 1976) and ability to hydrolyse urea (Kreger-van Rij, 1984) although there are exceptions in the latter reaction. Ascomycetes and basidiomycetes frequently reproduce in nature or in the laboratory in their anamorphic states, and therefore also possess anamorphic names in the Deuteromycotina. In many cases anamorphs have been linked with teleomorphs and the anamorph name is no longer used. Occasionally the connection between the two states is tenuous, with only a few isolates completing the life cycle and in such instances both names are retained.

Nucleic acid hybridization studies, oligonucleotide cataloguing and chemotaxonomic analyses have recently led to significant changes in the classification of Gram-positive, catalase-negative, facultatively anaerobic cocci, previously all included in the genus Streptococcus (Schleifer and Kilpper-Balz, 1987). Nucleic acid analyses had its origins in studies of the prokaryotic genome (Marmur et al., 1963), and hinges on the assessment of relatedness by determining the mean molar percentage of guanine-plus-cytosine (mol % G+C) in the nuclear DNA. Price et al. (1978) maintain that "...if two organisms are related, they must retain in their genomes, base sequences that are descendant from a common ancestral base sequence; closely related organisms will have retained a greater portion of base sequences in common that have highly diverged...". However, this approach would appear to avoid the

issue as to whether or not it is possible, in practice, to distinguish between or determine relative proportions of ancestral and non-ancestral nDNA base sequences (Van der Walt, 1987). Continual improvements in molecular techniques for the isolation of genetic probes will no doubt contribute to solving this.

Chemotaxonomy or chemosystematics is the study of the chemical variation in living organisms and the use of chemical characters to classify and identify. More specifically, chemotaxonomy is concerned with the discontinuous distribution of certain chemicals such as amino acids, sugars, lipids and proteins, so that relationships between species may be established (Goodfellow and Minnikin, 1985). Thus, the menaquinone composition of aerobic, endospore-forming bacteria and polar lipid patterns of staphylococci are uniform; but menaquinones and polar lipids are of value in the subgenic classification of Staphylococcus and Bacillus (Nahaie et al., 1984; Minnikin and Goodfellow, 1981).

The determination of fatty acid compositions has proved to be useful, particularly in the identification of various ascomycetous yeasts grown under standard conditions (Kock et al., 1986). The influence of culture age on the cellular long-chain fatty acid composition was used by Smit et al. (1987), to distinguish between three basidiomycetous yeasts.

Viljoen et al. (1988) grouped species from the genera Candida, Kluyveromyces and Saccharomyces according to their cellular long-chain fatty acid compositions, carbon source utilization patterns and degree of cell differentiation. They were able to establish possible teleomorph-anamorph relations and general similarities

between yeasts by the closeness of organisms within a group. Unrelated yeasts were positioned some distances from each other. Kock et al. (1988) used a similar approach for the genus Kluyveromyces and were able to propose a scheme for the evolutionary development of species within the genus. In both of the above cases, the presence of linolenic acid correlated with the formation of pseudomycelium and/or true hyphae as well as the utilization of a large number of carbon sources, whilst the absence of this acid correlated with a more rudimentary pseudomycelium and/or single cells and the utilization of only a few carbon sources.

The eventual delimitation of yeast species would seem to hinge on an integrated approach, as used by Kock et al. (1988), where many criteria based on morphological, sexual, genetic, physiological, biochemical and ecological features of as many strains as possible are taken into account (Van der Walt, 1987).

Computers have been used for storing taxonomic data and for constructing extensive taxonomic keys (Barnett et al., 1975; 1983). The use of such keys enables the identification of the yeast to be determined more quickly once all the physiological and morphological data have been entered. The National Collection of Yeast Cultures (NCYC) have developed a COMPuter ASSisted identification system ("COMPASS"), in which the properties of the yeast being tested are compared with those for all described taxa in "The Yeasts" (Kreger-van Rij, 1984). The system is based on methods developed by Lapage et al. (1970;1973) for bacteria. The advantage of the scheme is that only 46 of the numerous classical

tests are required for complete identification. The 46 characteristics of the unknown yeast are compared with a probability matrix so that each test result is compared with each record in the database and each record is given an identification score. The score represents the probability of the unknown yeast being a member of the taxon described in the record. The highest scoring taxa are sorted and listed. In this way no elimination of possible taxa is made on the basis of a single character, as is the case with keys which, because many isolates are frequently atypical, could lead to false identifications.

The classical taxonomic framework may be used to provide a contracted reference frame for a limited number of yeasts, for example: food spoilage yeasts, pathogenic yeasts, or those used for a special purpose such as the industrial yeasts used in brewing. Such a classification is artificial in that it is based on the deliberate selection of a limited number of criteria relevant to the particular purpose for which it is being used. However, in situations where a rapid identification is imperative, these schemes perform reliably, providing they are used solely for the yeast groups for which they were designed. Pathogenic yeasts are being isolated more frequently as their involvement in infections of immuno-suppressed patients is more commonly recognized. API (Analytab Products, Div. of Ayerst Laboratories, Plainview, N.Y.) have developed a system whereby the strain's ability to assimilate 20 carbon compounds is analysed to give an identification within 24, 48 or 72 hours, whilst also checking the morphology (Dermoumi, 1979; Land et al., 1979). These ready-made kits cater for a

restricted domain of around 50 species of clinical importance. Other keys specifically designed for the most important foodborne yeasts consider an even smaller number of species, for example, Rodrigues de Miranda (1984a)and Pitt and Hocking (1985).

A need for a simplified identification scheme for a wide range of foodborne yeasts was recognized by Deák and Beuchat (1987). They combined two approaches, first they reduced the number of tests by selecting the most efficient ones (Gower and Barnett, 1971), and second they reduced the number of yeasts by only considering those found in foods. This scheme was designed not only to aid routine identification, but also for the ecological study of foods where a large number of strains are concerned and where traditional identification procedures prove impractical owing to the number of tests and the incubation periods required. Indeed, this approach could be adopted to compile schemes to identify yeasts in particular foods, such as milk and dairy products or meats and meat products. Such studies will hopefully lead to a greater awareness of the contribution of yeasts to the microbial associations of foods. Consequently, good manufacturing practices (GMP), and an improvement in product quality may be attained as described in Chapter 1.

Yeast taxonomists are unable to study yeast populations in a natural habitat, unlike plant taxonomists. This has obvious limitations since the yeasts are responding to an artificial environment. Ecological studies must employ a different approach to that of classical identification, such as those used by Starmer et al. (1980) and Davenport (1981).

ECOLOGICAL

This alternative approach to yeast identification has a great advantage over classical identification when considering the possible roles of yeasts in their habitats. The species name does not usually reflect either the source or the role of an organism, whereas by using certain characteristics to form groups it is possible to show ecological traits between and within related genera (Davenport, 1985a).

Beech et al. (1968) developed a scheme for yeast identification using two keys. The first involved separation into groups based on colony appearance and is of particular use in splitting the many yeasts obtained in an ecological survey into more manageable groups with similar properties. The second involved biochemical tests designed to reflect the environment from which a yeast was isolated. In the food and beverage industry the principal types of spoilage caused by yeasts include formation of a film, a haze, or taints and/or odours as described in Chapter 1. Davenport (1985a) suggests using observations such as; film or pellicle formation, growth in osmophilic media and growth in the presence of preservatives, as parameters by which to recognize and gauge the importance of isolates from spoilage situations. An integrated environmental approach based on the above observations was used to assess yeast problems associated with raw materials, processing and packaging of selected beverages and foods (Davenport, 1987). Such simplified techniques enable an experienced worker to detect and recognize potential spoilage organisms within one to two weeks compared with one to two months using classical procedures. Another

and novel approach by Davenport (1980b, 1987) was to use commercial media, which were not intended for studies of yeasts, to highlight particular properties. For example, eosin methylene blue (Lab M) supported ascospore formation of Hansenula anomala but not of Hanseniaspora osmophila and gradient plates containing Bismuth Sulphite Agar (Oxoid) and Wort Agar (Oxoid) were used to detect hydrogen sulphide producing strains of Saccharomyces cerevisiae.

SCREENING AND ISOLATION

As discussed in the previous Chapter, in most cases yeasts form only a small part of the microbial association of a food when compared with the bacterial component. This has obvious repercussions in terms of screening and isolation. The latter was covered in Chapter 1 where it was concluded that the addition of antibiotics such as chloramphenicol and oxytetracycline, was preferable when compared with the acidification of media which frequently supported growth of lactic-acid bacteria (Koburger and Rodgers, 1978). Screening using specific media such as molybdate agar supplemented with 0.125% calcium propionate (Rale and Vakil, 1984) has been described for certain groups of yeasts. In this case yeasts from tropical fruits are differentiated using colony colour. Another example is that described by Andrews (1982) who used Wallerstein laboratory nutrient agar supplemented with 0.1% (w/v) Euchrysine 2GNX, to detect potential wine-spoilage yeasts after only 20-36 h incubation.

Electronic techniques have been widely employed for bacterial detection, particularly in the food industry, because they require

minimal sample preparation, provide continuous automatic monitoring and produce relatively rapid results (Wood and Gibbs, 1982; Firstenberg-Eden, 1983; Gibson et al., 1984; Easter and Gibson, 1985). In terms of yeast contamination, where growth is slow and numbers often low, such rapid and sensitive methods would confer a considerable advantage. Rapid detection of yeasts by impedance measurements has been investigated by Zindulis (1984) and Shapton and Cooper (1984). Schaertel et al. (1987) evaluated several mycological media for use in such a system and Henschke and Thomas (1988) used an impedance method to detect wine-spoilage yeasts. At present, appropriate media for yeast detection by conductance measurements are being examined by Connolly et al. (1988). It remains to be seen whether or not such techniques will be used for routine detection of yeasts in foods.

In order to investigate the ecology of yeasts from meats and poultry and their potential significance as spoilage organisms, a screening method for the analysis of large numbers of isolates was developed. By determining the most efficient tests for the delimitation of yeasts isolated from meats and poultry, a key was produced which allowed species identification using the minimum number of characteristics. Grouping, along the lines of Davenport (1985b) was used to identify particular characteristics of 'meat yeasts' based on their response to conditions found in their environment. Finally, observations on the formation of specialised structures, which possibly enable adaptation to a particular environment, are discussed in the relevant sections of Chapters 3 and 4.

MATERIALS AND METHODS

ISOLATION

Combinations of media, incubation temperature and incubation period were investigated in order to establish a protocol that would ensure maximum recovery of the greatest range of yeasts. Four media, Acidified Malt Extract Agar (Lab M), Acidified Plate Count Agar (Lab M), Oxytetracycline Glucose Yeast Extract agar (Oxoid) and Rose Bengal Chloramphenicol Agar (Oxoid), were incubated at 5°C, 15°C and 25°C. Six replicate plates of each medium incubated at 15°C and 25°C were examined at 5, 7 and 10 d, those incubated at 5°C were examined at 7, 10 and 14 d.

Samples of appropriate serial dilutions of meat suspensions (quarter strength Ringer's solution - Oxoid) and swab suspensions ('calgon' Ringer's solution - Oxoid) were spread-inoculated onto four replicate plates of Oxytetracycline Glucose Yeast Extract Agar (OGY) and incubated at 25°C for 7 d.

Colony Selection

Each colony was streaked onto Yeast Morphology Agar (Difco). When the number of colonies on a plate was too great (> 40) to isolate each one, then three representative colonies were taken.

The isolation plates were left inverted at room temperature to allow any slow-growing yeasts to develop and were examined later for morphological features including mycelium, pseudohyphae, ascospores, chlamydospores and teliospores.

Macromorphology

Colony shape, colour, size and texture were recorded together with the relative abundance of that particular colony type.

Ballistospore formation was observed either by an array of satellite colonies, a mirror image of ballistospores on the inner side of the Petri dish lid, or by growth on the upper and lower agar surface of a double layer plate (Van der Walt and Yarrow, 1984). The latter method was preferable, due to the constant humidity obtained by sealing the two Petri dishes with tape.

Micromorphology

Phase-contrast microscopy (x400) of wet mounts (distilled water) was used. Minimum and maximum length and breadth of cells were recorded using a microscope with a calibrated eyepiece graticule. Cell shape, cell arrangement and vegetative reproduction in the form of budding cells (monopolar, bipolar of multilateral) or fission cells (Van der Walt and Yarrow, 1984) were noted. The formation of pseudohyphae, true mycelium, arthrospores, ballistospores, ascospores, endospores, chlamydospores and teliospores were determined. A description of any sexual structures was made.

Photographs of the gross and microscopic yeast morphology were taken. In the latter case cells were immobilised by sealing wet mounts with nail-varnish.

PURIFICATION

The yeast from the primary isolation plate was streaked onto

Yeast Morphology Agar (YMA) to obtain well separated colonies. Single isolated colonies were purified by repeated replating (3 repeats) on YMA. After incubation at 25°C for 5 d the plate was examined carefully to ensure freedom from contaminants. If all colonies appeared to be the same, the culture was assumed to be pure and identification was attempted.

STORAGE

Duplicate cultures of the purified yeast were prepared on YMA slopes. After incubation at 25°C for 3 d, or until good growth was obtained, the stock cultures were stored at 4°C. One culture was retained solely for the preparation of new stocks; the other was the working culture.

Stock cultures were subcultured every six months.

For short-term storage (1-2 wk), the cultures could be stored in Petri dishes in the refrigerator, preferably in unsealed plastic bags.

IDENTIFICATION

A list of standard generic abbreviations may be found at the front of this thesis.

CLASSICAL

Yeast isolates were characterised by procedures described by Van der Walt and Yarrow (1984) and identified with definitions given by Kreger-van Rij (1984).

The following stock cultures were examined alongside new isolates in order to ensure that standard procedures were being followed. All those cultures followed by * are known to have a perfect state. Thus, throughout identification, any evidence for this state was recorded.

Candida famata* (NCYC 611), C. ingens* (NCYC 822), C. krusei* (NCYC 247), C. mesenterica (NCYC 390), C. rugosa (NCYC 130), C. stellata (NCYC 486), C. valida* (NCYC 327), C. zeylanoides (MRI 1945), Hansenula anomala (NCYC 711) and Rhodotorula rubra (CMI 38784).

Preparation of Inoculum

Stored cultures were subcultured twice on YMA at 25°C for 3-5 d to bring them to a state of active growth. This was followed by growth on starvation medium (Bacto Yeast Nitrogen Base (Difco) with 1.5% (w/v) purified agar (Oxoid) and 0.5% (w/v) glucose (A.R. grade, Fisons) for 5 d at 25°C. A yeast suspension was prepared using sterile distilled water and standardised by further dilution until black lines (0.5 mm thick and 5 mm apart) drawn on white card appeared as diffuse bands, a +2 growth was equivalent to approximately 10^6cells ml^{-1} (Wickerham, 1951; Anon., 1986).

One drop of the standard yeast suspension, delivered from a Pasteur pipette, was used to inoculate liquid media. Solid media were inoculated from the same suspension with an inoculating loop.

Macromorphology

Colony form, texture and colour on YMA was described after 5 d

at 25°C. Ballistospore formation and characteristic odour were recorded. After incubation at room temperature for a further 14 d, these characters together with the presence or absence of extracellular polysaccharide were noted.

Cultures were inoculated into 10 ml Yeast Morphology Broth (Difco) and incubated for 3 d at 25°C. The formation and appearance of a pellicle, islets, ring and/or sediment were recorded. These observations were repeated after a further 14 d incubation at room temperature.

Micromorphology

The yeast cells were examined in Yeast Morphology Broth (YMB) after 3 d at 25°C using phase-contrast microscopy (x400). Cell shape, minimum and maximum length and breadth of cells were recorded together with cell arrangement. Ballistospore and ascospore formation were also determined.

The formation of pseudohyphae, true mycelium, arthrospores, endospores, ascospores, ballistospores, chlamydospores and teliospores were observed by direct examination (x150) of growth on Cornmeal Agar (Oxoid) plates. 'Dalmau' plate (Van der Walt and Yarrow, 1984) were streak- and spot-inoculated with the standardised cell suspensions. The spot was covered with a sterile cover slip before incubation for 7, 14 and 21 d at 25°C. The edge of the streak (aerobic) and the spot ('anaerobic') were examined.

Sporulation was characterised by inoculation of presporulation medium (0.8% (w/v) Yeast Extract (Lab M), 0.3% (w/v) bacteriological peptone (Lab M), 10% (w/v) glucose (Fisons) and 2%

(w/v) agar No. 2 (Lab M)) and incubation for 5 d at 25°C, followed by inoculation of sporulation media and incubation for 3 d at 25°C. If the yeasts had not sporulated, they were maintained at room temperature and examined at weekly intervals for a further 4 weeks. The following media, incubated at 15°C, 20°C and 25°C for 3, 5, 7, 14 and 21 d, were examined for ability to induce ascospore formation:

1. Dilute V8 agar - filtered Campbell's V8 juice (Whatman No. 1 paper), adjusted to pH 5.5 with sodium hydroxide, diluted with distilled water from 1:2, 1:9, 1:19 to 1:29 and 2% (w/v) agar No. 2 (Lab M) was added, the mixture was steamed.
2. Gorodkowa agar (modified) - 0.1% (w/v) glucose (Fisons), 1% w/v) mycological peptone (Lab M), 0.5% (w/v) sodium chloride (Fisons) and 2% (w/v) agar No. 2 (Lab M) were added to tap water and autoclaved for 15 min at 1.03×10^5 Pa.
3. Fowell's acetate agar - 0.5% (w/v) sodium acetate trihydrate (BDH) was added to distilled water and adjusted to pH 6.8 with hydrochloric acid (BDH), 2% (w/v) agar No. 2 (Lab M) was added and autoclaved for 15 min at 1.03×10^5 Pa.
4. Potassium acetate agar - 1% (w/v) potassium acetate (BDH), 0.1% (w/v) yeast extract (Lab M), 0.05% (w/v) glucose (Fisons) and 2% (w/v) agar No. 2 (Lab M) were added to distilled water and autoclaved for 10 min at 6.89×10^4 Pa.
5. Cornmeal agar (Oxoid).

Dilute V8 agar (V8), Gorodkowa agar (GWA) and Fowell's acetate agar (FAA) are referred to by Van der Walt and Yarrow (1984) and

potassium acetate agar (PAA) is a modified version of that used by Anon. (1986).

For routine use potassium acetate agar, incubated at 25°C and examined at 3, 5, 7 d and then at weekly intervals for four weeks, was chosen.

Ascospore formation on potassium acetate agar (PAA) slopes and/or cornmeal agar (CMA) plates was assessed by phase-contrast microscopy (x400) of wet mounts. These revealed the maximum detail of spore morphology (e.g. spore number and shape). Ascospore formation was further confirmed by heat-fixing wet mounts which were stained by steaming with 5% (w/v) aqueous malachite green and counterstained with 0.5% (w/v) safranine (Van der Walt and Yarrow, 1984).

Fermentation of Carbohydrates

Inoculation onto yeast extract glucose agar (YEG) containing 0.5% (w/v) mycological peptone (Lab M), 0.5% (w/v) yeast extract (Lab M), 0.5% (w/v) glucose (Fisons), 1.5% (w/v) agar No. 2 (Lab M) and adjusted to pH 7.2 with sodium hydroxide, was followed by incubation for 3 d at 25°C. One drop of a +2 standardised suspension was prepared and transferred to each of the fermentation tubes.

Fermentation basal medium (Wickerham, 1951) contained 0.45% (w/v) yeast extract (Lab M) and 0.75% (w/v) mycological peptone (Lab M) dissolved in distilled water. Bromothymol blue was added to give a sufficiently dense green colour and 2 ml amounts were placed in 150 x 12 mm capped tubes containing Durham tubes (25 x 6 mm).

Glucose (A.R. grade Fisons), galactose, sucrose, maltose and lactose (A.R. grade BDH) were prepared (6% (w/v) in distilled water) and filter-sterilised (Sartorius Minisart filters, 0.2 μm) before adding (1 ml) to the fermentation tubes.

After inoculation the medium was covered with sterile paraffin oil, incubated at 25°C and examined for gas production and acid formation at 3, 7, 14 and 21 d.

Multipoint Inoculation

The following media inoculated, in duplicate, with 21 yeasts simultaneously using a Denley multipoint inoculator (Denley, Sussex), were incubated at 25°C and examined at 5 and 14 d (Dalton, 1984).

Assimilation of Carbon Compounds

An agar modification of the method described by Beech et al. (1968) and Dalton (1984) was used to determine ability to utilise carbon compounds. One part (10 ml) of filter sterilised 6.7% (w/v) yeast nitrogen base (Difco) and 5% (w/v) of the carbon source were added to 9 parts (90 ml) of 2.0% (w/v) sterile purified agar (Oxoid) at 45°C and poured into Petri dishes. The carbon sources tested at a final concentration of 0.5% (w/v) were adonitol, D-arabinose, L-arabinose, arbutin, cellobiose, citric acid, M-erythritol, galactitol, D-galactose, glucosamine hydrochloride, gluconate, M-inositol, inulin, lactose, maltose, mannitol, mannose, melibiose, α-methyl glucoside, rhamnose, ribose, salicin, sorbitol, sorbose, starch, succinate, sucrose, trehalose, xylitol and xylose.

Ethanol was used at a final concentration of 0.62% (w/v), glycerol 0.4% (w/v), melezitose 0.36% (w/v) and raffinose 1% (w/v). Inulin and starch were dissolved by gentle heating in 90 ml distilled water with 2% (w/v) purified agar. After autoclaving (15 min at 1.03×10^5 Pa), 10 ml of filter sterilised 6.7% (w/v) yeast nitrogen base (Difco) was added and the medium was poured into Petri dishes. A positive control containing glucose and a negative control with no carbon source were also included. All compounds were analytical grade reagents (Fisons, BDH or Sigma).

Assimilation of Nitrogen Compounds

An agar modification of the method described by Beech et al. (1968) and Dalton (1984) was used to determine a yeast's ability to utilise nitrogen compounds. One part (10 ml) of filter sterilised 11.7% (w/v) yeast carbon base (Difco) and 0.78% (w/v) potassium nitrate (A.R. grade Fisons), 0.26% (w/v) sodium nitrite (A.R. BDH), 0.64% (w/v) ethylamine hydrochloride (A.R. Aldrich Chemical Company Ltd.) or 0.5% (w/v) creatine (A.R. Sigma) were added to 9 parts (90 ml) of sterile 2% (w/v) purified agar (Oxoid and poured into Petri dishes. A negative control without a nitrogen source was also included.

Growth in Vitamin-Free Medium

A modification of the method described by Beech et al. (1968) was used to determine a yeast's ability to grow without an external source of vitamins. One part (10 ml) of a filter-sterilised solution containing 16.7% (w/v) vitamin free yeast base (Difco) was

added to 9 parts (90 ml) of sterilised purified agar (Oxoid) at 45°C, and poured into Petri dishes. After incubation at 25°C, plates were examined at 5, 7 and 14 d.

In order to ensure that any internal growth factors were depleted, it was necessary to subculture an organism which grew from the first plate to a second plate. This was done using a 'replicate-plate' method.

Growth at Different Temperatures

Yeast morphology agar (YMA) plates were inoculated in duplicate and incubated at 37°C for 5 d and 5°C for 14 d. In cases of weak growth a subculture was made and incubated for a further 5 d.

Lipolysis

The lipolytic activity was assessed in duplicate using tributyrin agar (Oxoid) in Petri dishes. Incubation was at 25°C and determination of the zone of clearing (mm) made at 3, 5 and 7 d.

Starch Formation

Plates used to assess assimilation of glucose were flooded with iodine after 7 d. A blue colouration of the colony indicated production of extracellular starch and a brown colouration extracellular glycogen.

Production of Urease

Urea agar base (Oxoid) was prepared and dispensed (9.5 ml) into universal bottles before autoclaving for 20 min at 1.03×10^5 Pa

and cooling to 45°C. A filter sterilised (0.5 ml) urea solution 40% (w/v) was aseptically added and the mixture allowed to set at a slant. After incubation at 25°C, slopes were examined for a pink colouration at 1, 2 and 3 d.

Growth on Sodium Chloride Medium

Yeast cultures were streaked across the surface of a medium containing 0.5% (w/v) yeast extract (Lab M), 5% (w/v) glucose (Fisons), 1% (w/v) mycological peptone (Lab M), 3% (w/v) agar No. 2 (Lab M) and supplemented with 5, 10, 15, 20 or 25% (w/v) sodium chloride (BDH). Four yeasts were inoculated on each plate, incubated at 25°C and examined at 3, 5 and 7 d.

Gelatin Hydrolysis

A 15% (w/v) solution of gelatin (Oxoid) was mixed with hot distilled water, dispensed (4.5 ml) into capped test tubes (150 x 12 mm) and autoclaved for 15 min at 1.03×10^5 Pa. After cooling to about 37°C, 0.5 ml of a solution containing 11.7% (w/v) yeast carbon base (Difco) and 5% glucose (Fisons) was added aseptically to the tubes which were allowed to set vertically. Incubation was at room temperature and the depth of the liquified gelatin measured in mm at 7, 14 and 21 d.

Cycloheximide Resistance

Capped tubes (150 x 12 mm) containing 4.5 ml distilled water were autoclaved for 15 min at 1.03×10^5 Pa. One gram or 0.1 g of cycloheximide (Sigma) was added to 2.5 ml acetone to give a

1000 ppm or a 100 ppm solution respectively. This was added to 6.7% yeast nitrogen base (w/v) and 10% glucose (w/v), and the solution mixed and filter sterilised before adding 0.5 ml to each of two test tubes. Tubes were incubated at 25°C with occasional agitation for 3 weeks. A positive result was recorded when +2/+3 growth occurred at 7 d and a slow or weak result if there was +2/+3 growth at 14 - 21 d.

Splitting of Arbutin

Arbutin (Sigma) was added (0.5%, w/v) to capped test tubes (150 x 12 mm) containing, 6 ml of 1% (w/v) yeast extract (Lab M) and 2% (w/v) agar No. 2 (Lab M). One drop of sterile 1% (w/v) ferric ammonium citrate (BHD) was added aseptically immediately after autoclaving (15 min at 1.0 x 10^5 Pa) and mixed carefully to avoid frothing before allowing the agar to set as a slant. Incubation was at 25°C with examination at 2, 5, 7 and 14 d. If arbutin was split, then a brown colour developed in the medium.

COMPASS

The tests used were prepared as described above except in the following cases: fermentation and assimilation of carbon compounds, assimilation of nitrogen compounds and growth in vitamin free media. All tests were incubated at 25°C for up to 21 d (Table 2 .2).

Fermentation of Carbon Compounds

Five grams (w/v) of yeast extract (Difco) dissolved in 1 litre of distilled water were dispensed (4.5 ml) into screw-capped Bijoux

Table 2.2 Recording tests for COMPASS

	Time in days			
Test	2	7	14	21
Cell Division	+			
Cultural Characteristics		+	+	+
Fermentation	+	+	+	+
Carbon Assimilation		+	+	+
Nitrogen Assimilation		+	+	+
Vitamin Free Growth		+	+	+
Urease Activity	+	+		
Vegetative Reproduction on Cornmeal Agar				+
Sexual Reproduction on Potassium Acetate Agar				+

bottles containing Durham tubes (25 x 6 mm). After autoclaving for 15 min at 6.89 x 10^4 Pa, 0.5 ml of the filter sterilised 20% (w/v) carbohydrate solutions was added.

After inoculation bottles were inverted to release any air trapped in the Durham tube, the screw caps were tightly closed and the bottles incubated at 25°C.

Examination for gas production was at 2, 7, 14 and 21 d; the screw caps were gently loosened so that any small amounts of gas production could be detected by a slight hissing noise or the release of small bubbles into the medium.

Assimilation of Carbon and Nitrogen Compounds

Solutions of basal medium containing yeast nitrogen or yeast carbon base and the relevant carbon or nitrogen source were prepared as previously described, filter sterilised and 1 ml was added to capped-tubes (150 x 12 mm) containing 9 ml of sterile distilled water. The caps were loose fitting to keep conditions as aerobic as possible.

Incubation was at 25°C with examinations at 7, 14 and 21 d for growth. It was measured by placing a well shaken tube against a card on which black lines were drawn (see preparation of inoculum). Growth was assessed as follows:

0 No growth - no turbidity; black lines clearly visible.

+1 Very weak growth - slight turbidity; black lines distinct but edges slightly blurred.

+2 Weak growth - black lines appear as diffuse bands.

+3 Good growth - no black lines or diffuse bands visible.

+2 and +3 growth was recorded as positive; +1 growth as negative unless the yeast was particularly slow growing.

All the following carbon compounds were analar grade (Fisons, BDH or Sigma): glucose, galactose, sorbose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, glycerol, erythritol, ribitol, galactitol, D-mannitol, D-sorbitol, α-methyl glucoside, salicin, lactic acid, succinic acid, citric acid and inositol.

Nitrogen compounds used were potassium nitrate (A.R. grade, Fisons) and ethylamine hydrogen chloride (A.R. grade, Aldrich Chemical Company Ltd.).

Growth in Vitamin-Free Medium

A filter-sterilised 16.7% (w/v) solution of vitamin-free yeast base (Difco) was added (1 ml) to bottles containing sterile distilled water (9 ml). Incubation was at 25°C for 7, 14 and 21 d. Growth was determined as for the assimilation tests. If growth occurred a further subculture into fresh medium was made from the first tube. A positive result was only recorded if +2 or +3 growth took place in the second tube.

Having done the 46 microbiological tests, the results were entered into the computer as follows: '0' - absent; '1' - present (weak or latent); '2' - present (strong); 'U' - unknown. Recommended values were a maximum of 2 unknowns and an identification score ≥ 0.9. If no single identification with a score = 0.9 was obtained, a list of taxa with scores which combined

to meet or exceed the score of 0.9 were listed.

As much additional information as possible was obtained (e.g. description of pellicle, formation of teliospores etc.), even though such results were not entered into the computer, so that any identifications suggested could be confirmed using 'The Yeasts - A Taxonomic Study' (Kreger-van Rij, 1984).

There were three confirmatory tests:

1. Growth in 10 (w/v) Sodium Chloride Medium

Peptone 1% (w/v), yeast extract 0.5% (w/v), glucose 2% (w/v) and sodium chloride 10% (w/v) were added to distilled water, dispenses (10 ml) into bottles and autoclaved for 15 min at 1.03 x 10^5 Pa. The bottles were examined for growth at 7, 14 and 21 d and scored as for the assimilation tests.

2. Growth in 60% (w/v) Glucose Medium

Glucose 60% (w/v), yeast extract 2.34% (w/v) and agar No. 2 (Lab M) 2.34% (w/v) dissolved in distilled water, were dispensed (10 ml) into bottles, autoclaved for 15 min at 6.89 x 10^4 Pa and allowed to set as slopes. Incubation was at 25°C and growth examined at 7, 14 and 21 d.

3. Growth in 1% (w/v) Acetic Acid Medium

Glucose 10% (w/v), tryptone 1% (w/v), yeast extract 1% (w/v) and agar No. 2 (Lab M) dissolved in distilled water was dispensed (10 ml) into bottles, autoclaved for 15 min at 1.03 x 10^5 Pa and allowed to cool to about 50°C. Glacial acetic acid (1% (w/v) was aseptically added to each of the bottles which were gently mixed and allowed to set as slopes. Incubation was at 25°C and growth determined at 7, 14 and 21 d.

ECOLOGICAL

Primary separation into groups was based on colony appearance after incubation for 5 d on the initial isolation medium. The following groups were noted: 1. pigmented and/or filamentous (dark, carotenoid, other); 2. mucoid; 3, smooth, rough, smooth/rough and 4. slow-growing (appeared after 5 d). The colony was streak- or loop-inoculated onto YMA, VRBG and YMB.

Secondary separation into the probable genus or genera was based on cell morphology (cell shape, type of vegetative reproduction, formation of pseudohyphae, true mycelium, arthrospores, ballistospores, ascospores and teliospores) and on simple physiological tests (production of urease, assimilation of nitrate, fermentation of glucose and assimilation of maltose, sucrose, melezitose, raffinose and galactose).

Tertiary separation into species or species group involved a combination of assimilation tests, other tests including growth at different temperatures, growth in 0.1% (w/v) cycloheximide, osmophilic tolerance and detailed morphological observations.

SCREENING

Screening Media

The following media were assessed for ability to support yeast growth whilst allowing differentiation between genera: cornmeal agar (Oxoid), wort agar (Oxoid), potato dextrose agar (Oxoid), eosin methylene blue agar (Lab M), violet red bile glucose agar (Lab M), mannitol salt agar (Oxoid) and MacConkey agar (Oxoid).

Colonies from primary isolation plates were streak-inoculated (four per plate), incubated at 25°C and examined at 3, 5, 7 and 12 d.

Screening at Isolation

Violet Red Bile Glucose Agar supplemented with oxytetracycline VRBGO (0.01% w/v) was used alongside Oxytetracycline Glucose Yeast Extract Agar (OGY) and Rose Bengal Chloramphenicol Agar (RBC) as a primary isolation medium for six different meat suspensions.

Ten grams of a range of meats were placed in sterile quarter-strength Ringer's solution (Oxoid) in a sterile stomacher bag and homogenised for 60 sec in a Colworth Stomacher 400 (Seward, London). The homogenate was poured into a sterile screw cap bottle and serial dilutions were prepared in quarter-strength Ringer's solution. Six replicate samples (0.1 ml) of appropriate dilutions were spread-inoculated and the plates incubated at 25°C for 7 d.

RESULTS

ISOLATION AND METHODOLOGY

To ensure maximum recovery of the greatest range of yeasts, the most appropriate isolation medium, incubation temperature and period of incubation were determined. Serial dilutions of meat homogenates were spread-inoculated (six replicates) onto acidified malt extract agar (MAA), acidified plate count agar (PCAA), oxytetracycline glucose yeast extract agar (OGY) and Rose Bengal chloramphenicol agar (RBC). Figure 2.1 shows isolation plates containing RBC, OGY and PAA incubated for 10 d at 25°C. Colonies were very small on the two acidified media and tended to 'run' on RBC agar. At a temperature of 5°C, no colonies were detected until 7 d and at higher temperatures there was overgrowth of colonies after 10 d (Table 2.3).

Counts were analysed in a 3-way ANOVA using 'Generalised Linear Modelling' (GLIM). Medium, temperature and period of incubation all exerted very highly significant ($P < 0.001$) effects (Table 2.4). Of the interaction effects, time and temperature, and temperature and medium were significant. This indicates that the effect of temperature and of medium, were not constant over the range of incubation times used.

To establish the most appropriate combination, differences between means for levels of each factor were analysed for significance (> 2 x standard error). Oxytetracycline glucose yeast extract agar, incubated at 25°C for 14 d were the ideal set of

Figure 2.1 The effect of medium on yeast colony morphology

A = acidified plate count agar

B = oxytetracycline glucose yeast extract agar

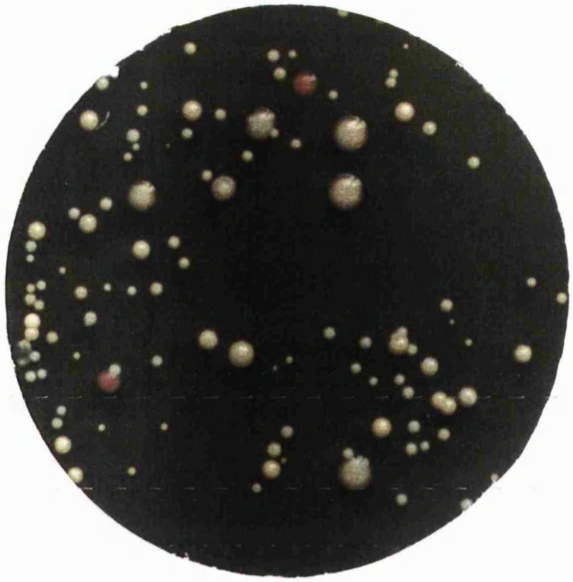
C = Rose Bengal chloramphenicol agar

Figure 2.1 The effect of medium on yeast colony morphology

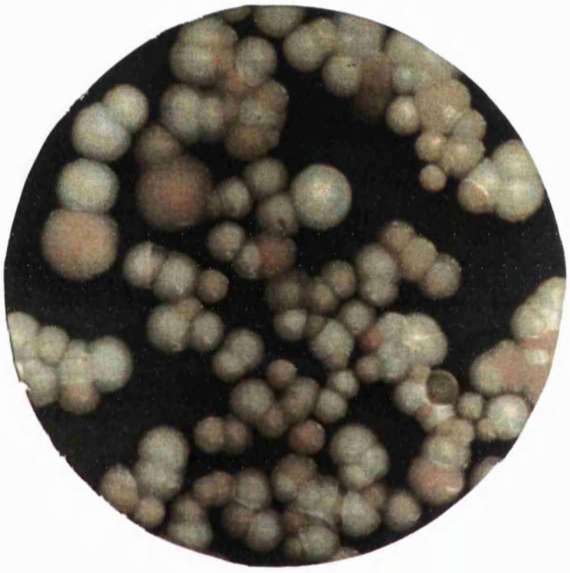
A = acidified plate count agar

B = oxytetracycline glucose yeast extract agar

C = Rose Bengal chloramphenicol agar



A



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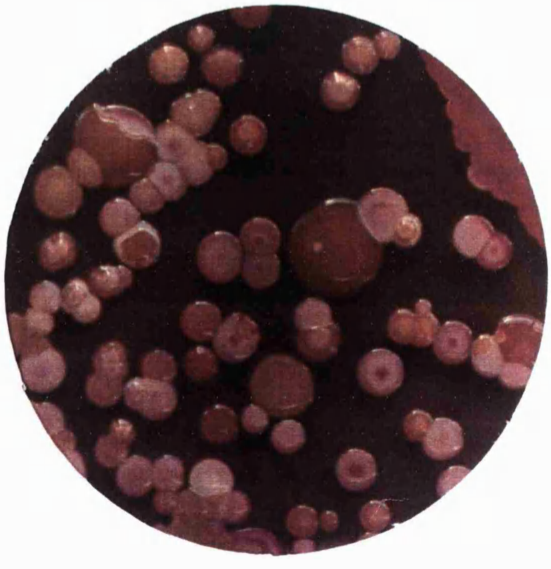


C

Table 2.3 Comparison of media, temperature and period of incubation for recovery of yeasts from meats*

Medium	MAA				PCAA				OGY				RBC				
Incubation	5	7	10	14	5	7	10	14	5	7	10	14	5	7	10	14	
5°C	NT	1.0	1.3	2.1	NT	1.3	1.5	1.8	NT	1.6	2.1	2.4	NT	1.2	1.6	1.9	$\bar{x}_5 = 1.65$
15°C	0.8	1.1	1.3	NT	1.3	1.7	1.9	NT	1.7	1.9	1.9	NT	1.6	1.7	1.9	NT	$\bar{x}_{15} = 1.57$
25°C	1.9	1.9	1.9	NT	1.9	1.9	1.8	NT	2.1	2.2	2.2	NT	1.9	2.1	2.1	NT	$\bar{x}_{25} = 1.99$
	$\bar{x}_M = 1.48$				$\bar{x}_P = 1.68$				$\bar{x}_O = 2.01$				$\bar{x}_R = 1.78$				$\bar{x}_5 = 1.65$ $\bar{x}_7 = 1.63$ $\bar{x}_{10} = 1.79$ $\bar{x}_{14} = 2.05$

* Counts = mean $\log_{10}$ cfu g^{-1} (6 replicates)

MAA = acidified malt extract agar

PCAA = acidified plate count agar

OGY = oxytetracycline glucose yeast extract agar

RBC = Rose Bengal chloramphenicol agar

$\bar{x}$ = mean values

Table 2.4 Analysis of Variance[1]

Source	df	SS	MS	F	P
Medium	3	7.755	2.585	18.300	***
Time	3	3.767	1.256	8.890	***
Temperature	2	11.53	5.765	40.828	***
Medium x Time	9	1.225	0.136	0.960	NS
Medium x Temp.	6	2.500	0.417	2.950	**
Time x Temp.	3	1.209	0.403	2.850	*
Medium x Time x Temp.	9	0.585	0.065	0.460	NS
Error	180	25.418	0.141		

1. Analysis according to Sokal and Rohlf (1981).

P values = significance. *** = $P < 0.001$, ** = $P < 0.01$,

* = $P < 0.05$, NS = $P > 0.05$.

conditions. However, the significant interaction effect of time and temperature was due to an increase in counts with time at 5 and 15°C but not at 25°C, probably because at the higher temperature, counts were already at a maximum on day 5. For this reason isolation medium was incubated at 25°C and examined after 5-7 d.

It was apparent that the nature of the work would determine the methodology used. In the present study large numbers of yeasts were isolated and time, materials and incubator space were limited. For this reason, assimilation of a range of compounds was determined using multipoint-inoculation of solid agar rather than individual inoculation of test tubes containing liquid medium.

Fermentation was assessed by observing gas and acid production. Some non-fermentative species of Trichosporon and Candida caused a change in pH (detected by colour change of indicator) and slight fermentation even though they were unable to assimilate the compound. This phenomenon was noted by Davenport (1975) who suggested that some yeasts, particularly those of soil and plant origin, metabolised the indicator and fermented the small amount of ethanol used as a solvent. In fact, very few of the yeasts isolated in this study (Chapters 3 and 4) were fermentative and any positive results were repeated in medium without indicator.

After testing media containing a range of tweens and other fat sources, Tributyrin Agar (Oxoid) was chosen to determine lipolytic activity as it was easily obtainable and results were reproducible because the composition of the medium was assured.

IDENTIFICATION

Classical and ecological identification methods were evaluated for their utility to aid investigation of yeast ecology in meats and poultry.

CLASSICAL

The accuracy of the procedure devised in collaboration with Dr. R.R. Davenport for the identification of yeasts included in this study was assessed. Ten named cultures and 10 new isolates were examined in parallel. Based on 59 properties, named cultures (A-J) were identified correctly according to their catalogue name (Table 2.5). The names assigned to the new isolates (K-T) were therefore also presumed to be correct (Table 2.6). In addition, 63 isolates were characterised on two occasions to determine the extent, if any, of within laboratory variations in the assimilation tests. A percentage difference in the two sets of results of 1.13 was observed. This was well below the 5% level recommended by Sneath and Johnson (1972) for acceptable test reproducibility.

As well as an assessment of assimilation, further emphasis was given to ascospore formation (Table 2.7). Six yeasts were studied at 15°C, 20°C and 25°C on the following media: dilute V8 agar (V8), Gorodkowa agar (GWA), Fowell's acetate agar (FAA), potassium acetate agar (PAA) and cornmeal agar (CMA). Sporulation, which was only observed at 25°C, was recorded at either 7 d or 21 d. Debaryomyces hansenii sporulated on all the media given above and ascospores were observed in less than 7 d on GWA and PAA. With the

Table 2.5 Identification of stock cultures and new isolates

	Yeast Name	
	Identification	Perfect*
A	*Candida famata*	*Debaryomyces hansenii*
B	*Candida ingens*	*Pichia humboldtii*
C	*Candida krusei*	*Issatchenkia orientalis*
D	*Candida mesenterica*	
E	*Candida rugosa*	
F	*Candida stellata*	
G	*Candida valida*	*Pichia membranaefaciens*
H	*Candida zeylanoides*	
I	*Hansenula anomala*	
J	*Rhodotorula rubra*	
K	*Debaryomyces hansenii*	
L	*Candida lipolytica*	*Saccharomycopsis lipolytica*
M	*Candida tenuis*	
N	*Cryptococcus albidus*	*Filobasidium floriforme*
O	*Candida zeylanoides*	
P	*Trichosporon pullulans*	
Q	*Cryptococcus laurentii*	
R	*Trichosporon cutaneum*	
S	*Sporidiobolus salmonicolor*	
T	*Pichia etchellsii*	

*If the yeast was identified as an imperfect state of a recognized perfect state (Kreger-van Rij, 1984), then the perfect name is also given.

Table 2.6 Accuracy of Classical Identification Scheme[1]

Test	Stock Culture[2]										New Isolate									
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
Morphology																				
Pseudohyphae	−	+	+	+	+	−	+	+	−	−	−	−	+	−	+	+	−	+	+	+
True mycelium	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	+	+	−
Arthrospores	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−
Ballistospores	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Ascospores	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	+
Pellicle	+	+	+	−	+	−	+	−	−	−	+	+	+	−	−	−	−	+	+	−
Carotenoid pigment	−	−	+	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−
Fermentation																				
Glucose	−	−	+	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−
Assimilation																				
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Adonitol	+	−	−	+	−	−	−	+	+	+	+	−	+	+	+	+	+	−	+	−
D-arabinose	+	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	+	+	+	−
L-arabinose	+	−	−	−	−	−	−	−	−	+	−	−	−	+	−	+	+	+	−	−
Arbutin	+	−	−	+	−	−	−	+	+	+	+	−	+	+	−	+	+	+	+	+
Cellobiose	+	−	−	+	−	−	−	−	+	+	+	−	+	+	−	+	+	+	+	+
Citric acid	+	−	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
Erythritol	+	−	−	+	−	−	−	−	+	−	+	+	+	−	−	+	+	−	−	−

Table 2.6 continued.

Test	Stock Culture[2]										New Isolate									
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
Assimilation (continued)																				
Ethanol	+	+	+	+	+	−	+	−	+	+	+	+	+	+	−	+	−	+	+	+
Galactitol	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+	−	−
D-galactose	+	+	−	−	+	−	−	−	+	+	+	−	+	+	−	+	+	+	+	+
Glucosamine-HCl	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	+	−	−
Gluconic acid	+	−	−	−	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−
Glycerol	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	−	+	+	+	+
Inositol	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+	−	−
Inulin	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Lactic acid	+	+	+	+	+	−	+	−	+	+	+	+	+	−	−	−	−	+	−	+
Lactose	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	−	−
Maltose	+	−	−	+	−	−	−	−	+	+	+	−	+	+	−	+	+	+	+	+
Mannitol	+	−	−	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	−	−	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	−	−	−	−	−	−	−	−	−	+	−	−	+	−	+	+	+	−	−
Melezitose	+	−	−	−	−	−	−	−	+	+	+	−	+	+	−	+	+	+	−	+
α-M-glucoside	+	−	−	+	−	−	−	−	+	−	+	−	+	+	−	+	+	+	−	+
Raffinose	+	−	−	−	−	+	−	−	+	+	+	−	−	−	−	+	+	+	−	−
Rhamnose	+	−	−	−	−	−	−	−	−	−	+	−	+	+	−	+	+	−	−	−
Ribose	+	−	−	+	−	−	−	−	+	+	+	−	+	−	−	−	+	+	−	−
Salicin	+	−	−	+	−	−	−	+	+	+	+	−	+	+	−	+	+	+	+	+
Sorbitol	+	−	−	+	+	−	−	+	−	+	+	+	+	+	+	+	+	+	+	+
Sorbose	+	−	−	+	+	−	−	+	−	+	+	−	−	+	+	+	−	+	+	+

Table 2.6 continued

Test	Stock Culture[2]										New Isolate									
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
Assimilation (continued)																				
Starch	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	–	+	–	–
Succinic acid	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	–	–	+	–	+	–	–	+	+	+	–	+	+	–	+	+	+	+	+
Trehalose	+	–	–	+	–	–	–	+	+	+	+	–	+	+	+	+	+	+	+	+
Xylitol	+	–	–	+	+	–	–	–	+	+	+	–	+	+	–	–	+	–	+	+
Xylose	+	–	–	–	–	–	–	–	+	+	+	–	+	+	–	+	+	+	+	+
Ethylamine-HCl	+	+	+	+	+	–	+	+	+	+	+	+	+	–	+	+	+	+	+	+
Nitrate	–	–	–	–	–	–	–	–	+	–	–	–	–	+	–	+	–	–	+	–
Nitrite	+	–	–	–	–	–	–	+	+	–	+	–	–	+	+	+	–	+	+	–
Creatine	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+
Growth																				
Vitamin free	+	+	+	–	–	–	+	+	+	–	+	–	–	–	+	+	+	–	+	–
10% (w/v) NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–
37°C	–	+	+	–	+	–	–	–	+	–	–	+	–	–	–	–	–	+	–	+
0.01% (w/v) cycloheximide	–	–	–	–	–	–	–	+	–	–	+	+	+	–	+	–	+	+	+	–
0.1% (w/v) cycloheximide	+	+	–	–	–	–	–	+	–	–	+	+	–	–	+	–	+	+	+	–

Table 2.6 continued

Test	Stock Culture[2]										New Isolate									
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
Production																				
Starch	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-
Hydrolysis																				
Urea	-	+	-	-	-	-	-	-	-	+	-	+	-	+	-	+	+	+	+	-
Arbutin	+	-	-	+	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+
Gelatin	-	-	-	+	+	-	+	+	-	+	-	+	-	-	-	+	-	-	+	-
Lipolysis																				
Tributyrin	+	-	+	+	+	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+

1. Devised in collaboration with Dr. R. R. Davenport.

2. Stock cultures were from NCYC and new isolates from skinless sausage.

Table 2.7 Ascospore formation on a range of media

Yeast Genera Species	Medium V8*	GWA	FAA	PAA	CMA
Debaryomyces hansenii	+	++	+	++	+
Hansenula anomala	+	-	-	+	+
Pichia burtonii	-	+	-	+	+
etchellsii	-	-	-	+	+
membranaefaciens	+	-	-	+	+
Saccharomycopsis lipolytica	-	-	-	+	+

* V8 = Dilute V8 agar; GWA = Gorodkowa agar; FAA = Fowell's acetate agar; PAA = potassium acetate agar; CMA = cornmeal agar.

++ = ascospore formation 7d.

+ = ascospore formation 21d.

- = no ascospore formation

other yeasts sporulation, which took more than 7 d, occurred on only 2 or 3 of the media. As a result of this survey, potassium acetate agar and cornmeal agar were adopted to study a yeast's ability to form ascospores. Those which sporulated in less than 7 d were scored as ++ and those in less than 21 d were scored as +. If there was no sporulation at this stage cultures were maintained for a further week after which sporulation was scored as +/- and no sporulation as -. Ascospore formation was assessed using phase contrast microscopy (x 400) and confirmed using a malachite green staining procedure.

COMPASS

This COMPuter-ASSisted identification scheme uses only 46 tests and was devised for rapid identification of yeasts in industrial laboratories.

Ten isolates identified by the classical method described above (Table 2.8) were used with the COMPASS system. Data were entered into the computer and a yeast name and identification score recorded on results sheets (Table 2.9). In only four cases were the same names obtained from both procedures and in three of these, COMPASS listed an alternative name. Scores of 0.94 were given to yeast identifications which were obviously incorrect based on simple observations such as pigmentation. Where the yeast was identified correctly (Sk/O/7: Tricosporon cutaneum) the identification score was only 0.69 and no alternative name was given. It was decided therefore that the COMPASS system was inappropriate for this study and that a simplified classical scheme

Table 2.8 Identification of yeasts by COMPASS

Isolate Code	Identification	
	Classical*	COMPASS
SK/R/9	*Debaryomyces hansenii*	*Cryptoccus laurentii*
SK/O/8	*Candida lipolytica*	*Trichosporon cutaneum*
SK/R/2	*Candida tenuis*	*Candida famata* *Cryptococcus laurentii*
SK/R/16	*Cryptococcus albidus*	*Cryptococcus albidus* *Cryptococcus terreus*
SK/O/3	*Candida zeylanoides*	*Zygosaccharomyces rouxii*
SK/R/18	*Trichosporon pullulans*	*Trichosporon pullulans* *Candida humicola*
SK/O/1	*Cryptococcus laurentii*	*Cryptococcus laurentii* *Cryptococcus luteolus*
SK/O/7	*Trichosporon cutaneum*	*Trichosporon cutaneum*
SK/O/6	*Sporidiobolus salmonicolor*	*Leucosporidium antarticum*
SK/O/5	*Pichia etchellsii*	*Candida multis-gemmis* *Candida parapsilosis*

* Classical procedure devised in collaboration with

Dr. R.R. Davenport.

Table 2.9 COMPASS yeast identification results sheets

Yeast Identification Results Sheet

Present (weak or latent) = 1; Present (strong) = 2; Absent = 0

Yeast Name *Trichosporon cutaneum* ID Score 0.6900052

Yeast Strain Number SK/O/7

Morphological Tests:

Pellicle	2
Fission	0
Pseudomycelium	2
True mycelium	2
Ballistospores	0
Arthrospores	0
Ascospores	0

Physiological Tests

Fermentation:

Glucose	0
Galactose	0
Sucrose	0
Maltose	0
Lactose	0

Assimilation:

Glucose	2	Inulin	1	Galactitol	2
Galactose	2	Starch	0	D-mannitol	2
Sorbose	2	D-xylose	2	D-sorbitol	0
Sucrose	2	L-arabinose	1	α-M-glucoside	2
Maltose	2	D-arabinose	2	Salicin	2
Cellobiose	2	D-ribose	0	Lactic acid	2
Trehalose	2	L-rhamnose	0	Succinic acid	2
Lactose	2	Ethanol	2	Citric acid	2
Melibiose	1	Glycerol	2	Inositol	2
Raffinose	2	Erythritol	0	Potassium nitrate	0
Melezitose	2	Ribitol	0	Ethylamine HCl	2

Growth in vitamin-free medium	2
Urease activity	0

Table 2.9 continued

Yeast Identification Results Sheet

Present (weak or latent) = 1; Present (strong) = 2; Absent = 0

Yeast Name *Leucosporidium antarticum* ID Score 1.000000

Yeast Strain Number SK/O/6

Morphological Tests:

Pellicle	2
Fission	0
Pseudomycelium	2
True mycelium	2
Ballistospores	2
Arthrospores	0
Ascospores	0

Physiological Tests

Fermentation:

Glucose	0
Galactose	0
Sucrose	0
Maltose	0
Lactose	0

Assimilation:

Glucose	2	Inulin	0	Galactitol	0
Galactose	2	Starch	0	D-mannitol	0
Sorbose	0	D-xylose	2	D-sorbitol	0
Sucrose	0	L-arabinose	0	α-M-glucoside	0
Maltose	2	D-arabinose	0	Salicin	2
Cellobiose	2	D-ribose	0	Lactic acid	0
Trehalose	2	L-rhamnose	0	Succinic acid	0
Lactose	0	Ethanol	2	Citric acid	0
Melibiose	0	Glycerol	2	Inositol	0
Raffinose	0	Erythritol	0	Potassium nitrate	2
Melezitose	0	Ribitol	0	Ethylamine HCl	0

Growth in vitamin-free medium	2
Urease activity	2

Table 2.9 continued

Yeast Identification Results Sheet

Present (weak or latent) = 1; Present (strong) = 2; Absent = 0

Yeast Name Zygosaccharomyces rouxii ID Score 0.9480000

Yeast Strain Number SK/O/3

Morphological Tests:

Pellicle	1
Fission	0
Pseudomycelium	2
True mycelium	0
Ballispores	0
Arthrospores	0
Ascospores	0

Physiological Tests

Fermentation:

Glucose	0
Galactose	0
Sucrose	0
Maltose	0
Lactose	0

Assimilation:

Glucose	2	Inulin	0	Galactitol	0
Galactose	0	Starch	0	D-mannitol	2
Sorbose	2	D-xylose	0	D-sorbitol	2
Sucrose	1	L-arabinose	0	α-M-glucoside	0
Maltose	0	D-arabinose	0	Salicin	0
Cellobiose	0	D-ribose	0	Lactic acid	0
Trehalose	2	L-rhamnose	0	Succinic acid	2
Lactose	0	Ethanol	0	Citric acid	2
Melibiose	0	Glycerol	2	Inositol	0
Raffinose	0	Erythritol	0	Potassium nitrate	0
Melezitose	0	Ribitol	0	Ethylamine HCl	2

Growth in vitamin-free medium	1
Urease activity	0

would be preferable.

SIMPLIFIED CLASSICAL KEY

On the basis of data collected from the literature for 114 yeast species isolated from meat and poultry (Appendix A), the efficiency of various assimilation, fermentation and physiological tests was calculated according to Gower and Barnett (1971) viz.

$$e = (p - 0.5) + (q - 0.5) + r$$

where p was the ratio of species with positive responses, q the ratio of species with negative responses and r the ratio of variable responses. The most efficient test was that for which the value e was the smallest.

The assimilation of maltose was the most efficient test overall, e = 0.033. Although there were no variable responses with urease hydrolysis, the efficiency of this test was relatively low because the yeasts were divided into two unequal groups (Table 2.10).

The construction of the key therefore began with maltose assimilation which split the 114 species into two groups containing 73 positive and 54 negative yeasts - the 13 variable species were added to each group. The next most efficient test was selected for each branch in the key. Urease hydrolysis was used for maltose-positive and sucrose assimilation for maltose-negative species. The key was continued successively until eight main groups had been formed (Figure 2.2). Only 6 tests were required to place a yeast into a particular group (Table 2.11). By following the relevant Group key (Table 2.12), a further 6 tests were used to identify a

Figure 2.2 Master key for identification.

Three petri dishes and three test tubes are required for identification of one strain.

*Figures denote the number of species contained in each group.

Assimilation tests: M = maltose, Mz = melezitose, S = sucrose, NO_3 = nitrate; R = raffinose, G = galactose, d = fermentation of glucose, U = urease hydrolysis and cyc = growth in the presence of 0.01% (w/v) cycloheximide.

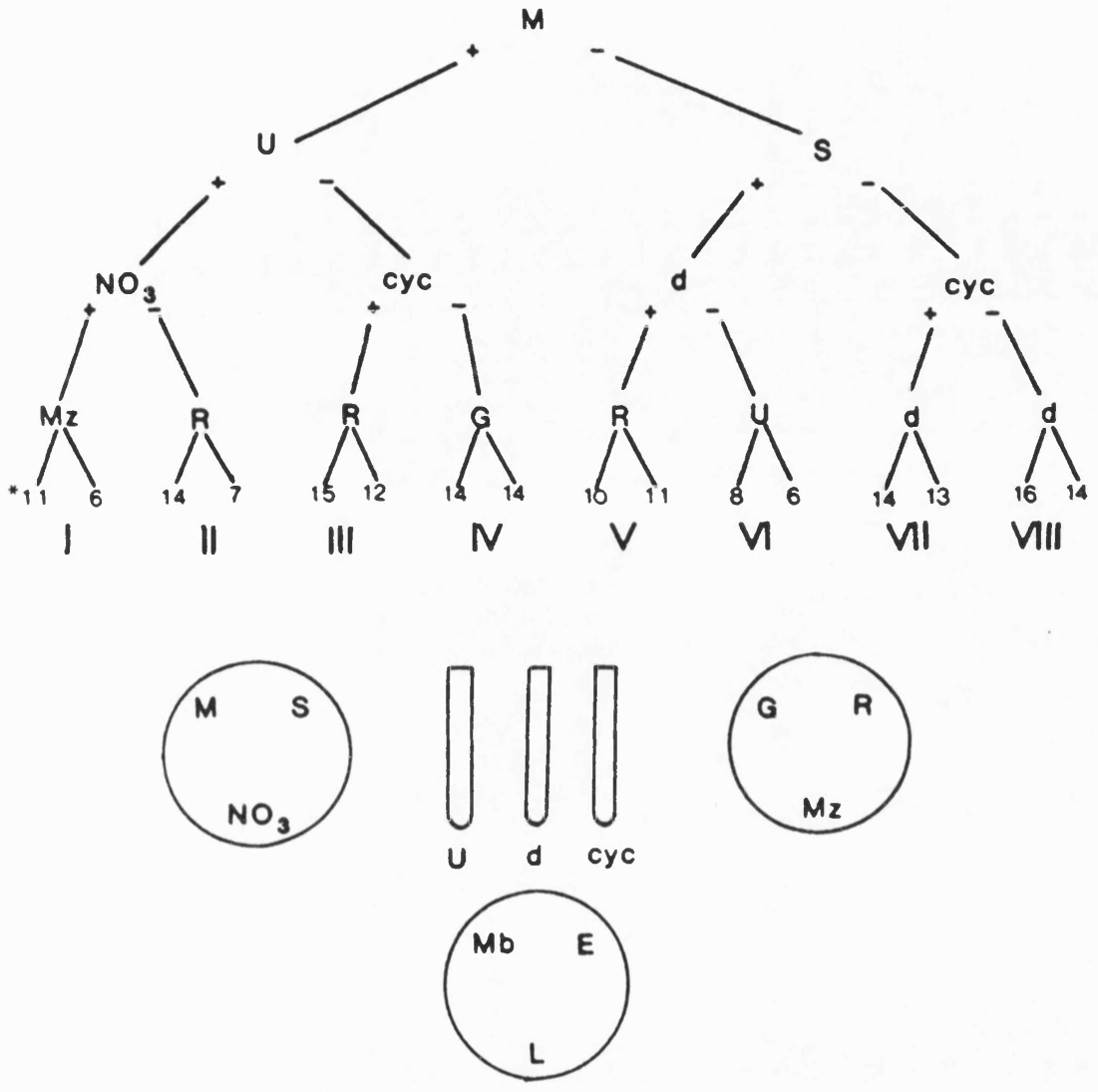

M
U
S
NO3
cyc
d
cyc
Mz
R
R
G
R
U
d
d
*11 6
14 7
15 12
14 14
10 11
8 6
14 13
16 14
I
II
III
IV
V
VI
VII
VIII
M S
NO3
U d cyc
G R
Mz
Mb E
L

Table 2.10 Efficiency of tests used to identify meat and poultry yeasts

Test	Response[1] +	-	v	Efficiency[2]
Maltose	73	54	13	0.033
Sucrose	78	48	12	0.051
Melezitose	63	73	22	0.059
Glucose fermentation	68	71	25	0.073
Raffinose	57	78	21	0.075
0.01% Cycloheximide	72	68	26	0.079
Urease	34	80	0	0.081
Erythritol	40	84	10	0.086
Nitrate	27	86	1	0.134
Galactose	85	65	36	0.165
Lactose	33	81	20	0.208
Melibiose	23	106	15	0.291

[1] A total of 114 different yeasts have been isolated from meat and poultry (Appendix A).

[2] Efficiency was calculated according to Gower and Barnett (1971).

Table 2.11 A simplified key for characterisation of yeasts isolated from meat and poultry

Key to Groups

1.	Maltose +	2
	Maltose -	5
2.	Urease +	3
	Urease -	4
3.	Nitrate +	Group I
	Nitrate -	Group II
4.	Cycloheximide +	Group III
	Cycloheximide -	Group IV
5.	Sucrose +	6
	Sucrose -	7
6.	Glucose fermentation +	Group V
	Glucose fermentation -	Group VI
7.	Cycloheximide +	Group VII
	Cycloheximide -	Group VIII

Table 2.12 Group keys for simplified identification

Character	Positive	Negative
Group I	(Maltose$^+$, Urease$^+$, nitrate$^+$, 15 species)	
1. Pigmented[1]	2	7
2. Ballistoconidia[2]	3	4
3. Cycloheximide	*Sporidiobolus ruinenii salmonicolor*	*Sporobolomyces puniceus roseus*[3]
4. Melezitose	5	*Rhodotorula graminis*
5. Starch production	6	*Rhodotorula glutinis*
6. Erythritol	*Cryptococcus macerans*	*Rhodosporidium infirmominiatum*
7. Sucrose	8	12
8. Arthroconidia	*Trichosporon pullulans*	9
9. Ballistoconidia	*Bullera tsugae*	10
10. True hyphae	*Leucosporidium scotti*	11
11. Pseudomycelium	*Candida aquatica*	*Cryptococcus albidus*
12. Melezitose	*Candida buffonii*	*Candida etchellsii*
Group II	(Maltose$^+$, Urease$^+$, Nitrate$^-$, 17 species)	
1. Arthroconidia	2	3

2.	Sarcina-like agglomerates of cells are formed by septation in different planes of individual blastospores or of cells infilaments	*Trichosporon inkin*	*Trichosporon cutaneum*
3.	Pigmented	**4**	**9**
4.	Erythritol	**5**	**6**
5.	Starch production	*Cryptococcus hungaricus*	*Cryptococcus flavus*
6.	Ballistoconidia	**7**	**8**
7.	Growth at 37°C	*Sporobolomyces albo-rubescens*	*Sporidiobolus*[4] *pararoseus* *Sporobolomyces roseus*
8.	Cycloheximide	*Rhodotorula rubra*	*Cryptococcus hungaricus*
9.	Ballistoconidia	*Bullera alba*	**10**
10.	Erythritol	**11**	**17**
11.	Pseudohyphae	**12**	**13**
12.	Melibiose	*Candida humicola*	*Candida curvata*
13.	Starch production	**14**	*Cryptococcus flavus*
14.	Growth at 37°	**15**	**16**
15.	Melibiose	*Cryptococcus laurentii*	*Cryptococcus neoformans*
16.	Lactose	*Cryptococcus laurentii*	*Cryptococcus luteolus*
17.	Lactose	**18**	**19**
18.	Pseudohyphae	*Candida curvata*	*Cryptococcus gastricus*
19.	Melezitose	**20**	*Filobasidium capsuligenum*

20.	Sucrose	21	*Cryptococcus gastricus*
21.	Growth at 37°C	*Cryptococcus neoformans*	*Filobasidium uniguttulatum*

Group III (Maltose$^+$, Urease$^-$, Cycloheximide$^+$, 22 species)

1.	Raffinose	2	20
2.	Melezitose	3	8
3.	Erythritol	4	11
4.	Glucose fermentation	5	6
5.	True hyphae	*Pichia burtonii*	6
6.	Cells oval, long-oval or cyclindrical after 3d at 25°C.	*Debaryomyces polymorphus*	7
7.	Ascospores oval not round	*Debaryomyces marama*	*Debaryomyces hansenii*
8.	Erythritol	9	10
9.	Glucose fermentation	*Pichia burtonii*	*Candida ciferrii*
10.	Nitrate	*Candida versatilis*	*Candida kefyr*
11.	Growth at 37°C	12	16
12.	Usually conjugated ascus, containing one warty ascospore.	*Debaryomyces hansenii*	13
13.	Melibiose	*Pichia guilliermondii*	14
14.	Pseudomycelium	1	*Candida haemulonii*
15.	True mycelium	*Candida steatolytica*	*Candida inositophila*
16.	Lactose and melibose	17	18
17.	A dull creeping pellicle but no ring after 3d at 25°C.	*Debaryomyces castellii*	*Debaryomyces hansenii*

18.	A thick creamy, wrinkled pellicle after 3d at 25°C	*Candida intermedia*	**19**
19.	Ascus usually formed by bud-mother cell conjugation. One warty ascospore with a small lipid inclusion. Released ascospores do not tend to agglutinate	*Debaryomyces hansenii*	*Kluyveromyces thermotolerans*
20.	Glucose fermentation	**21**	**29**
21.	Nitrate	**22**	**23**
22.	Erythritol	*Hansenula polymorpha*	*Candida versatilis*
23.	Erythritol	*Candida tenuis*	**24**
24.	Sucrose	**25**	**28**
25.	Pseudohyphae	**26**	*Candida haemulonii*
26.	A ring and islets after 3d at 25°C	**27**	*Candida diddensiae*
27.	Pseudomycelium consist of blastoconidia arranged singly, in short chains and in clusters but not in verticils	*Candida tropicalis*	*Candida steatolytica*
28.	Cells ovoid or elongate with a pear or sausage - like shape, not globose or short ovoid.	*Candida catenulata*	*Candida tropicalis*
29.	Sucrose	**30**	**32**
30.	Growth at 37°C	*Candida blankii*	**31**
31.	Melezitose	*Debaryomyces marama*	*Candida glaebosa*
32.	Erythritol	*Pichia media*	*Candida catenulata*

Group IV (Maltose$^+$, Urease$^-$, Cycloheximide$^-$, 24 species)

1.	Galactose	**2**	**22**
2.	Erythritol	**3**	**8**
3.	Glucose fermentation	**4**	**7**
4.	Nitrate	*Hansenula anomala*	**5**
5.	Raffinose	**6**	*Candida tenuis*
6.	A sediment, ring and possibly a dry pellicle after 3d at 25°C.	*Debaryomyces hansenii*	*Candida silvicultrix*
7.	Ascospores oval not round	*Debaryomyces mamama*	*Debaryomyces hansenii*
8.	Raffinose	**9**	**14**
9.	Arthroconidia	*Trichosporon fennicum*	**10**
10.	Spores	**11**	*Candida intermedia*
11.	Glucose fermentation	**12**	**13**
12.	Conjugation or protruberances	*Torulaspora delbrueckii*[5] *Debaryomyces hansenii*	*Saccharomyces cerevisiae*
13.	Pseudohyphae	*Pichia carsonii*	*Debaryomyces hansenii*
14.	Glucose fermentation	**15**	**21**
15.	Spores	**16**	**19**
16.	Conjugation or protruberances	**17**	*Saccharomyces cerevisiae*
17.	Protruberances	*Torulaspora delbrueckii*	**18**
18.	Melezitose	*Pichia etchellsii*	*Zygosaccharomyces rouxii*
19.	Pseudohyphae	*Candida sake*	**20**

20. Pulcherrima pigment	*Metschnikowia** *pulcherrima*	*Metschnikowia reukaufii*
21. Growth at 37°C	*Pichia etchellsii*	*Pichia carsonii*
22. Erythritol	**23**	**26**
23. Sucrose	**24**	*Candida molischiana*
24. Nitrate	*Hansenula anomala*	**25**
25. Melbiose	*Candida mesenterica*	*Candida ernobii*
26. Nitrate	**27**	**29**
27. Growth at 37°C	**28**	*Candida melinii*
28. Raffinose	*Candida globosa*	*Hansenula californica*
29. Spores	**30**	**33**
30. Conjugation or protruberances	**31**	*Saccharomyces cerevisiae*
31. Protruberances	*Torulaspora delbrueckii*	**32**
32. Melezitose	*Pichia rhodanensis*	*Zygosaccharomyces rouxii*
33. Sucrose	**34**	*Candida insectamans*
34. A thin, delicately wrinkled pellicle after 3d at 25°C	*Candida solani*	*Metschnikowia reukaufii*

Group V (Maltose$^-$, Sucrose$^+$, Glucose fermentation$^+$, 16 species)

1. Raffinose	**2**	**15**
2. Galactose	**3**	**10**
3. Nitrate	**4**	**5**
4. Urease	*Candida curiosa*	*Candida magnoliae*

5.	Cycloheximide	**6**	**8**
6.	Growth at 37°C	*Candida kefyr*	**7**
7.	Spores	*Saccharomyces exiguus*	*Candida gropengiesseri*
8.	Spores	**9**	*Candida gropengiesseri*
9.	Protruberances	*Torulaspora delbrueckii*	*Saccharomyces cerevisiae* $_6$ *exiguus*
10.	Cycloheximide	**11**	**12**
11.	Nitrate	*Candida magnoliae*	*Torulaspora globosa*
12.	Spores	**13**	**14**
13.	Protruberances	*Torulaspora delbrueckii*	*Saccharomyces cerevisiae*
14.	Nitrate	*Candida magnoliae*	*Candida apicola* (glycerol$^+$) *stellata* (glycerol$^-$)
15.	Erythritol	**16**	**17**
16.	Galactose	*Pichia farinosa*	*Candida ernobii*
17.	Galactose	**18**	**22**
18.	Nitrate	*Candida magnoliae*	**19**
19.	Spores	**20**	*Candida parapsilosis*
20.	Conjugation or protruberances	**21**	*Saccharomyces cerevisiae* $_6$ *exiquus*
21.	Protruberances	*Torulaspora delbrueckii*	*Zygosaccharomyces rouxii*
22.	Spores	**23**	**26**
23.	Nitrate	*Hansenula californica*	**24**

24.	Conjugation or protruberances	**25**	*Saccharomyces cerevisiae*
25.	Protruberances	*Torulaspora delbrueckii*	*Zygosaccharomyces rouxii*
26.	Nitrate	*Candida magnoliae*	*Candida diversa*

Group VI (Maltose$^-$, Sucrose$^+$, Glucose fermentation$^-$, 14 species)

1.	Nitrate	**2**	**6**
2.	Urease	**3**	**5**
3.	Erythritol	*Trichosporon pullulans*	**4**
4.	Ballistoconidia	*Sporidiobolus salmonicolor*	*Rhodotorula glutinis*
5.	Cycloheximide	*Candida vanderwaltii*	*Wickerhamiella domercqii*
6.	Urease	**7**	**11**
7.	Lactose	**8**	**10**
8.	Arthroconidia	*Trichosporon cutaneum*	**9**
9.	Pseudomycelium	*Candida curvata*	*Cryptococcus dimennae*
10.	Raffinose	*Rhodotorula rubra*	*Rhodotorula minuta*
11.	Erythritol	*Pichia farinosa*	**12**
12.	Galactose	**13**	*Candida apicola*
13.	Growth at 37°C and pseudomycelium	*Candida kefyr*	*Candida gropengiesseri*

Group VII (Maltose$^-$, Sucrose$^-$, Cycloheximide$^+$, 22 species)

1.	Glucose fermentation	**2**	**14**

2.	Galactose	**3**	**11**
3.	Erythritol	**4**	**5**
4.	Nitrate	*Candida boidinii*	*Pichia farinosa*
5.	Nitrate	**6**	**8**
6.	Urease	*Candida etchellsii*	**7**
7.	A ring and possibly islets or a pellicle after 3d at 25°C	*Candida magnoliae*	*Candida wickerhamii*
8.	Arthroconidia	*Trichosporon fermentans* (cellobiose$^+$) *Geotrichum candidum* (cellobiose$^-$)	**9**
9.	Spores	*Saccharomyces dairensis*	**10**
10.	Islets and creeping growth up sides of tube after 3d at 25°C	*Candida catenulata*	*Candida zeylanoides*
11.	Erythritol	*Candida cantarellii*	**12**
12.	Growth at 37°C (or 42°C)	*Candida glabrata*	**13**
13.	1 to 2 spherical spores, not 2–4 hat-shaped spores normally liberated at maturity	*Hanseniaspora uvarum*	*Hanseniaspora valbyensis*
14.	Urease	**15**	**19**
15.	Nitrate	**16**	**18**
16.	Pigmented	*Sporidiobolus salmonicolor*	**17**
17.	Growth at 37°C	*Candida foliorum*	*Candida etchellsii*
18.	Arthroconidia	*Trichosporon cutaneum*	*Saccharomycopsis lipolytica*
19.	Erythritol	**20**	**21**

20.	Galactose	*Pichia farinosa*	*Candida pinus*
21.	Nitrate	*Candida vanderwaltii*	**22**
22.	Arthroconidia	**23**	**24**
23.	Growth at 37°C (and 42°C)	*Trichosporon capitatum*	*Geotrichum candidum*
24.	Spores	*Pichia humboldtii*	**25**
25.	Islets and creeping growth up sides of tube after 3d at 25°C	*Candida catenulata*	*Candida zeylanoides*

Group VIII (Maltose$^-$, Sucrose$^-$, Cycloheximide$^-$, 23 species)

1.	Glucose fermentation	**2**	**16**
2.	Nitrate	**3**	**5**
3.	Urease	*Candida etchellsii*	**4**
4.	Pseudomycelium	*Candida norvegica*	*Candida magnoliae*
5.	Erythritol	*Pichia farinosa*	**6**
6.	Spores	**7**	**14**
7.	Conjugation or protruberances	**8**	**11**
8.	Protruberances	*Torulaspora delbrueckii*	**9**
9.	Thin or thick, dry climbing pellicle after 3d at 25°C	**10**	*Zygosaccharomyces rouxii*
10.	Strong glucose fermentation	*Pichia fermentans*	*Pichia membranaefaciens*
11.	Growth at 40°C	*Issatchenkia orientalis*	**12**
12.	Cells large (3-10)x(4.5-21) µm not small (3-4.5)x(5.5-8.5) µm	*Saccharomyces cerevisiae*	**13**

13. Galactose	*Saccharomyces dairensis*	*Saccharomyces telluris*
14. Pseudomycelium	**15**	*Candida glabrata*
15. Growth at 40°C	*Issatchenkia orientalis*	*Candida*[7] *silvae*
16. Nitrate	**17**	**20**
17. Urease	**18**	**19**
18. Growth at 37°C	*Candida foliorum*	*Candida etchellsii*
19. Pseudomycelium	*Candida norvegica*	*Wickerhamiella domercqii*
20. Galactose	**21**	**25**
21. Urease	*Trichosporon cutaneum*	**22**
22. Erythritol	**23**	**24**
23. Growth at 37°C	*Pichia farinosa*	*Pichia haplophila*
24. Raffinose	*Candida apis*	*Candida*[8] *rugosa*
25. Spores	*Pichia membranaefaciens*	**26**
26. Raffinose	*Candida apis*	**27**
27. Growth at 42°C	*Candida inconspicua*	**28**
28. Pseudomycelium	**29**	*Candida silvatica*
29. Pseudomycelium consists of branched chains of cylindrical cells of similar size to the ovoidal blastoconidia they bear, giving a 'bushy' formation	*Candida vini*	*Candida*[7] *silvae*

Notes for Table 2.12

[1] Old cultures of *Cryptococcus albidus* may be very slightly pigmented.

[2] *Sporobolomyces puniceus* often failed to exhibit ballistospore production resulting in *Rhodosporidium infirmominiatum*. The former is palerose cf the latter which is grenadine pink.

[3] *Sporobolomyces roseus* produced ballistospores which appeared as satellite colonies, it did not produce starch.

[4] *Sporobolomyces roseus* produced ballistospores abundantly thus forming satellite colonies; it did not produce hyphae or teliospores.

[5] *Torulaspora delbrueckii* was cellobiose^{-ve}; commonly formed 2 spores/ascus; spores were smooth not warty. Protruberances not conjugation.

[6] *Saccharomyces cerevisiae* - cells large (5.0-10.0)x(5.0-12.0) μm
Saccharomyces exiguus - cells small (2.5-5.0)x(3.5-6.5) μm

[7] Pseudohyphae consisted of ramified chains bearing clusters of roundish blastoconidia in verticillated positions.

[8] Pseudohyphae was primitive and very branched, blastospores, if formed at all, were scarce.

• *Metschnikowia* are generally associated with nectars and flowers Meat may have become contaminated from hides and lairage.

yeast to species level. Thus, 12 tests together with simple macro- and micromorphological observations were sufficient to identify a yeast. In all cases, yeast names obtained using this key correlated exactly with those previously identified using the full classical procedure. The tests were done with a minimum of apparatus. Three Petri dishes (maltose, sucrose, nitrate, galactose, raffinose, melezitose, melebiose, erythritol and lactose assimilation) and three test tubes (urease hydrolysis, glucose fermentation and growth in 0.01% (w/v) cycloheximide), were all that were required (Figure 2.2). When a large number of isolates were to be tested then the multi-point inoculation technique was used to reduce the number of Petri dishes needed.

ECOLOGICAL KEY

This key which involved colony descriptions and simple tests was used successfully in conjunction with the classical key to identify yeasts isolated from meat and poultry. It has been extended to include all those species (114) mentioned in the literature to date (Appendix A).

Colony appearance on the isolation media (Table 2.13) was used to separate isolates into four groups. A key was devised to separate the pigmented and/or filamentous yeasts (Table 2.14). Some of the pigmented filamentous yeasts were included in Groups 1 and 2 because often the colonies were smooth initially and became rough subsequently. Group 3, which contained the greatest number of yeasts, was divided into those which appeared smooth with an entire or uneven margin and those which were smooth initially becoming

Table 2.13 Primary selection into four groups

Group	Genus	Colony Morphology
		PIGMENTED and/or FILAMENTOUS
1	*Aureobasidium*	
	Cladosporium	Dark, filamentous
	Geotrichum	
	Trichosporon	
	Saccharomycopsis	Cream, filamentous
	Pichia	
	Cryptococcus	Dark, non-filamentous
	Rhodosporidium	
	Sporidiobolus	Carotenoid
	Sporobolomyces	
	Rhodotorula	
2		**MUCOID**
	Bullera	
	Cryptococcus	
	Filobasidium	
	Leucosporidium	
3		**SMOOTH and/or ROUGH**
	Candida	
	Hanseniaspora	
	Hansenula	Smooth with entire or uneven
	Saccharomyces	margin
	Torulaspora	
	Zygosaccharomyces	
	Candida	
	Debaryomyces	
	Kluyveromyces	Smooth, striated becoming
	Pichia	rough or rough with entire,
	Saccharomycopsis	uneven or filamentous margin
	Trichosporon	
4		**SLOW-GROWING**
	Candida	
	Kloeckera	Caulifower or shell-like
	Kluyveromyces	

Table 2.14 Key 1 - Pigmented and/or filamentous colonies

	Character	Response +	Response -
1	Pigmented (not carotenoid)	2	3
2.1	Filamentous, colonies light: cream, grey, pink, brown becoming darker with maturity: green, brown, black	*Aureobasidium* *Cladosporium*	2.2
2.2	Filamentous, little colour change with maturity, arthrospores present	*Trichosporon* *Geotrichum*	2.3
2.3	Colonies become brown or black, no mycelium and extremely mucoid	*Cryptococcus*	2.4
2.4	Colonies yellow/orange on VRBG	*Saccharomyces*	*Pichia*
3.1	Shades of yellow, orange, pink and red; mucous growth; often pseudohyphae and true mycelium; may produce starch	*Rhodosporidium*	3.2
3.2	Ballistospores produced	3.3	3.4
3.3	Colonies normally dull, fringed with mycelium and soft, becoming tough	*Sporidiobolus*	*Sporobolomyces*
3.4	No mycelium; many strains mucous but others pasty, dry and wrinkled	*Rhodotorula*	*Sporobolomyces*

striped or rough with an entire, uneven or filamentous margin on further incubation. No yeasts were found belonging to Group 4, the slow-growing yeasts, but as certain species of Candida, Kloeckera and Kluyveromyces are known to take around 7-10 d to develop into 'cauliflower' colonies, these were included.

A second key based on simple diagnostic features (Table 2.15), was used to separate the four groups into genera. Group 1 was best differentiated using pellicle formation, cell shape, formation of arthrospores, ballistospores, teliospores and/or ascospores; Group 2 by using formation of pseudo- or true mycelium, ballistospores and/or teliospores; Group 3 by formation of pseudo- or true mycelium and assimilation of a mixture of compounds which indicated a smooth-becoming-rough colony rather than a smooth colony; Group 4 could be divided using cell shape, type of budding and ascospore formation.

Tertiary separation into species or group of species was achieved by doing more detailed morphological, physiological and biochemical analysis (Table 2.16). The ability to assimilate inositol, nitrate, cellobiose, to ferment glucose and to grow in the presence of 0.01% (w/v) cycloheximide and at 37°C were of particular use at this stage.

The key was adapted where appropriate to include tests which reflected the environment from which the yeast was isolated. Thus, ability to grow on sodium chloride medium, produce acetaldehyde and lipolyse tributyrin were used at the tertiary stage for yeasts isolated from sausage.

Table 2.15 Key 2 - Secondary Separation into Probable Genus or Genera

Genera	Group	Cells		B	P	Ps/T	Ar	Ac	Bl	T	U	NO_3	f	ASM	Comments
		O	O												
		O	o												
Aureobasidium	1	+	+	MPB	-	+	-	-	-	-	+	v	-	+	Many colony forms, may be dry or shiny, pink becoming black, soft becoming rough.
Cladosporium	Dark/Fil	+	+	MPB	-	v	-	-	-	-	?	?	-	-	Grey, green, black, aerial mycelium
Geotrichum		+	+	MPB	v	+	+	-	-	-	-	-	-	-	Little colour change, septa and chain formation independent of budding.
Trichosporon		+	+	MPB	+	+	+	-	-	-	v	v	v	v	Cell shape variable.
Saccharomycopsis	Cream/Fil	-	+	MPB	+	+	-	v	-	-	v	-	-	-	*S. lipolytica* is Urease-positive.
Pichia		+	+	MPB	+	+	-	+	-	-	-	-	v	+	Creeping pellicles.
Cryptococcus	Dark	+	+	MPB	-	-	-	-	-	-	+	v	-	+	Dark, thick cell walls.
Rhodosporidium		-	+	MPB	-	+	-	-	-	+	+	+	-	+	Clamp connections, colonies tend to be mucoid.
Sporidiobolus	Carotenoid	-	+	MPB	-	v	-	-	+	+	+	+	-	+	Clamp connections, colonies tend to be dull or powdery
Sporobolomyces		+	+	MPB	-	v	-	-	+	-	+	v	-	+	No clamp connections.
Rhodotorula		+	+	MPB	-	v	-	-	-	-	+	v	-	+	Most species show yeast cells only.
Bullera	2	-	+	MPB	-	-	-	-	+	-	+	v	-	+	Colonies may be cream and brown. Ballistospores are symmetrical.
Cryptococcus		+	+	MPB	-	-	-	-	-	-	+	v	-	+	Cream or pinkish, never myceliated.
Filobasidium	Mucous	+	+	MPB	-	+	-	-	-	-	+	v	-	+	May see conjugating cells, basidiospores, clamp connections.
Leucosporidium		+	+	MPB	-	+	-	-	-	+	+	+	v	+	Often isolated from environments $\leq 12°C$ and may have a mycelial periphery.

Table 2.15 Key 2 - Secondary Separation into Probable Genus or Genera

Genera	Group	Cells		B	P	Ps/T	Ar	Ac	Bl	T	U	NO_3	f	ASM	Comments
Aureobasidium	1 Dark/Fil	+	+	MPB	-	+	-	-	-	-	+	v	-	+	Many colony forms, may be dry or shiny, pink becoming black, soft becoming rough.
Cladosporium	Dark/Fil	+	+	MPB	-	v	-	-	-	-	?	?	-	-	Grey, green, black, aerial mycelium
Geotrichum	Dark/Fil	+	+	MPB	v	+	+	-	-	-	-	-	-	-	Little colour change, septa and chain formation independent of budding.
Trichosporon		+	+	MPB	+	+	+	-	-	-	v	v	v	v	Cell shape variable.
Saccharomycopsis	Cream/Fil	-	+	MPB	+	+	-	v	-	-	v	-	-	-	*S. lipolytica* is Urease-positive.
Pichia	Cream/Fil	+	+	MPB	+	+	-	+	-	-	-	-	v	+	Creeping pellicles.
Cryptococcus	Dark	+	+	MPB	-	-	-	-	-	-	+	v	-	+	Dark, thick cell walls.
Rhodosporidium	Dark	-	+	MPB	-	+	-	-	-	+	+	+	-	+	Clamp connections, colonies tend to be mucoid.
Sporidiobolus	Carotenoid	-	+	MPB	-	v	-	-	+	+	+	+	-	+	Clamp connections, colonies tend to be dull or powdery
Sporobolomyces	Carotenoid	+	+	MPB	-	v	-	-	+	-	+	v	-	+	No clamp connections.
Rhodotorula	Carotenoid	+	+	MPB	-	v	-	-	-	-	+	v	-	+	Most species show yeast cells only.
Bullera	2	-	+	MPB	-	-	-	-	+	-	+	v	-	+	Colonies may be cream and brown. Ballistospores are symmetrical.
Cryptococcus		+	+	MPB	-	-	-	-	-	-	+	v	-	+	Cream or pinkish, never myceliated.
Filobasidium	Mucous	+	+	MPB	-	+	-	-	-	-	+	v	-	+	May see conjugating cells, basidiospores, clamp connections.
Leucosporidium	Mucous	+	+	MPB	-	+	-	-	-	+	+	+	v	+	Often isolated from environments < 12°C and may have a mycelial periphery.

Table 2.15 continued

Genera		Group	Cells		B	P	Ps/T	Ar	Ac	Bl	T	U	NO_3	f	ASM	Comments
			OO / O o													
Candida		3	+	−	MPB	−	−	−	−	−	−	−	v	v	−	No pseudomycelium.
Hanseniaspora	Smooth, entire, uneven		O	+	BPB	−	v	−	+	−	−	−	−	+	−	Ascospores hat- or helmet-shaped.
Hansenula			+	+	MPB	+	+	−	+	−	−	−	+	v	v	Ester production.
Saccharomyces			+	+	MPB	v	−	−	+	−	−	−	−	+	v	Film may be formed.
Torulaspora			+	−	MPB	+	v	−	+	−	−	−	−	+	−	Protruberances on cells may be seen.
Zygosaccharomyces			+	+	MPB	v	−	−	+	−	−	−	−	+	−	Film may be formed; conjugation of cells precedes ascospore formation, ascospores are smooth.
Candida	Smooth, striated, rough, fil		+	+	MPB	+	+	−	−	−	−	v	v	v	+	Always pseudomycelium.
Debaryomyces			+	+	MPB	+	−	−	+	−	−	−	−	+	+	Ascospores warty, 1 per ascus
Kluyveromyces			+	+	MPB	−	+	−	+	−	−	−	−	+	+	May be slow-growing.
Pichia			+	+	MPB	+	+	−	+	−	−	−	−	v	v	Creeping pellicles.
Saccharomycopsis			+	+	MPB	v	+	−	v	−	−	v	−	v	−	*S. lipolytica* is Urease-positive.
Trichosporon			+	+	MPB	+	+	+	−	−	−	v	v	v	v	May form endospores.
Candida	Slow-growing	4	+	+	MPB	−	v	−	−	−	−	−	−	+	−	Often develop a brown tinge and an acidic odour; growth > 7 days.
Kloeckera			O	+	BPB	−	−	−	−	−	−	−	−	+	−	
Kluyveromyces			O	+	BPB	−	+	−	+	−	−	−	−	+	−	

Cells: OO = circular/ellipsoidal, oO = oval/elongate, O = ogive; B: Budding, MPB = multipolar budding, BPB = bipolar budding;

P = pellicle; Ps/T = pseudohyphae and/or true mycelium; Ar = arthrospores; Ac = ascospores; Bl = ballistospores; T = teliospores;

U = urease; NO_3 = nitrate; f = fermentation of glucose; ASM = assimilate a mixture of carbon compounds.

+ = growth; − = no growth; v = variable growth; ? = unknown

Table 2.16 Key 3 - Tertiary Separation into Species or Species Group*

Genera Species	Group	ASM	M	S	Mz	R	G	C	In	NO_3	f	Cx	5°	37°	L	Gel	NaCl	Morphology
Aureobasidium																		
pullulans	1	+	+	+	+	+	+	+	-	v	-	v	+	-	-	-	-	Mycelium mostly immersed. Slime indicates 1° conidia budding to form 2° conidia.
Geotrichum																		
candidum		-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	Hyphae fragment into unicellular units.
Trichospon																		
capitatum		-	-	-	-	-	v	-	-	-	-	+	-	+	-	+	-	Rugose, soft and agar digesting.
cutaneum		v	v	v	v	v	+	+	v	-	-	v	+	v	+	+	+	Light brown on VRGB.
pullulans		v	v	+	v	+	+	+	+	+	-	v	+	-	+	+	+	Starch formation.
Saccharomycopsis																		
lipolytica		-	-	-	-	-	v	v	-	-	-	+	+	v	+	+	+	Septa have no plasmodesmata or dolipore. Blastospores in small chains or verticils on the hyphae.
Pichia																		
burtonii		+	+	+	v	+	+	+	-	-	+	+	+	v	-	+	+	Blastospores may be borne on denticles.
Rhodosporidium																		
infirmominiatum		+	+	+	+	+	v	+	+	+	-	v	+	-	-	-	+	Teliospores are terminal or intercalary with granular bodies, some have thick walls.
Sporidiobolus																		
ruinenii		+	+	+	-	+	+	+	-	+	-	+	+	-	-	+	+	Homothallic. Hyphae have clamp connections and teliospores.
salmonicolor		v	v	v	v	v	v	v	-	+	-	+	+	-	-	+	+	Kidney-shaped ballistospores on cornmeal agar.
Sporobolomyces																		
roseus		+	+	+	+	+	v	v	-	-	-	v	+	-	-	+	+	No mycelium, orange/pink. Ballistospores on simple or branched sterigmata, terminally or laterally positioned.

Table 2.16 continued

Genera Species	Group	ASM	M	S	Mz	R	G	C	In	NO_3	f	Cx	5°	37°	L	Gel	NaCl	Morphology
Rhodotorula																		
glutinis		+	+	+	+	v	v	v	–	+	–	v	+	v	–	+	+	Cells ovoidal, globose, may be pseudomycelium.
minuta		+	–	+	+	–	v	+	–	–	–	v	+	v	v	+	+	Grows on 15% (w/v) Nacl.
rubra		+	v	+	v	+	v	v	–	–	–	v	+	v	–	+	+	Coral red to orange.
Bullera																		
alba	2	+	+	+	+	+	+	+	+	–	–	–	+	–	–	–	+	Starch formation.
tsugae		+	+	+	+	–	–	+	+	+	–	v	+	–	–	–	+	No starch formation.
Cryptococcus																		
albicus		+	+	+	+	v	+	+	+	+	–	–	+	–	+	+	+	May see basidial formation. starch formation.
hungaricus		+	+	+	+	+	+	+	+	–	–	–	+	–	+	v	+	Colonies tend to be reddish.
laurentii		+	+	+	+	+	+	+	+	–	–	v	+	v	+	+	+	Growth is slimy, may see conjugated cells and hyphae.
Filobasidium																		
capsuligenum		+	+	v	–	–	v	+	+	–	–	v	+	–	+	+	+	Clamp connections at cross-walls. Mycelium from conjugated yeast cells. Basidiospores terminal in petal-like whorl.
Leucosporidium																		
scottii		+	+	+	+	+	v	v	–	+	–	v	+	–	–	–	+	May see conjugating cells with developing hyphae. Thick-walled teliospores develop metabasidium and spores.
Candida																		
krusei	3	–	–	–	–	–	–	–	–	–	+	–	+	+	–	v	+	Colony becomes slightly wrinkled, has irregular edge and may be myceliated.
magnoliae		–	–	v	–	v	v	–	–	+	+	v	+	+	–	v	+	Cells (1.5-5)x(2-5) μm.
vini		–	–	–	–	–	–	–	–	–	–	–	+	–	+	v	+	Ovoid, cylindrical cells (2.9-4.3)x(5.8-10.1) μm.
zeylanoides		–	–	–	–	–	v	v	–	–	v	+	+	v	+	v	+	Mycelium feather-like, cells small, oval, elongate. Rarely fermentation
Torulaspora																		
globosa		–	–	+	–	+	–	–	–	–	+	+	+	+	v	v	+	Off-white to dark cream colonies, smooth and glossy, no mycelium.

Table 2.16 continued

Genera Species	Group	ASM	M	S	Mz	R	G	C	In	NO_3	f	Cx	5°	37°	L	Gel	NaCl	Morphology
Candida																		
boidinii	3	+	-	-	-	-	+	-	-	+	+	+	+	v	+	+	+	Cells long ovoid, cylindrical and slightly curved.
cantarellii		+	-	-	-	-	-	-	-	-	+	+	+	-	+	v	+	Cells globose.
catenulata		+	v	-	-	-	+	-	-	-	v	+	+	+	+	+	+	Sausage-, pear-like cells, grey-white colony.
curvata		+	v	+	v	+	+	+	v	-	-	v	+	v	+	+	+	Urease hydrolysis.
guilliermondii		+	+	+	+	+	+	+	-	-	+	+	+	+	v	v	+	Pseudohyphae, whorls, blastospores.
humicola		+	+	+	v	v	v	v	v	-	-	v	+	-	+	+	+	Urease hydrolysis and starch formation.
ingens		v	-	-	-	-	+	-	-	-	-	+	+	-	v	v	+	Dull, wrinkled pellicle. Cylindrical, large cells (4-8) x (8-16) μm.
inositophila		+	+	+	+	+	+	+	+	-	+	+	+	+	-	v	+	Abundant pseudomycelium, elongate cells, ovoid blastospores.
intermedia		+	+	+	+	+	+	+	-	-	+	v	+	-	-	-	+	Thick, wrinkled pellicle.
parapsilosis		+	-	+	+	-	+	-	-	-	+	v	+	+	+	-	+	Pseudomycelium consists of branched chains of elongated cells and sparse clusters of oval spores.
rugosa		v	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	Primitve pseudomycelium, very few blastospores - if at all.
steatolytica		+	+	+	+	v	+	+	+	-	+	+	+	+	-	v	+	Thick pellicle, extensive mycelial border.
tenuis		+	+	+	+	-	+	+	-	-	+	v	+	-	+	-	+	Texture membraneous. Bare, thin, wavy pseudomycelium.
Debaryomyces																		
hansenii		+	+	+	+	+	+	v	-	-	v	v	+	v	v	-	+	Mother-daughter conjugation precedes ascospore formation.
Pichia																		
carsonii		+	+	+	+	v	+	v	-	-	-	-	+	-	-	-	+	Thin to moderately heavy pellicles.
etchellsii		+	+	+	+	-	+	+	-	-	v	-	+	+	-	-	+	No pellicle.
fermentans		v	-	v	-	-	+	v	-	-	v	v	+	+	v	v	+	Growth is tannish yellow.

• Group compiled from species isolated in this study. ASM = assimilate a mixture of compounds; M = maltose, S = sucrose, Mz = melezitose, R = raffinose, G = galactose, C = cellobiose, In = inositol, NO_3 = nitrate, f = fermentation of glucose, Cx = growth in presence of cycloheximide (0.01% w/v), 5° = growth at 5°C, 37° = growth at 37°, L = lipolysis of tributyrin, Gel = hydrolysis of gelatine, NaCl = growth in presence of NaCl (10% w/v). + = positive, - = negative, v = variable.

SCREENING

Of the media used in an initial survey to investigate differential screening, one in particular, Violet red bile glucose agar (VRBG), was highly suitable (Table 2.17). Not only did it support the growth of the 'meat yeasts' but types of yeast could be differentiated on the basis of colour and texture.

Figure 2.3 clearly shows the differences between five yeasts: Candida zeylanoides, Debaryomyces hansenii, Cryptococcus albidus, Rhodotorula rubra and Candida lipolytica. The colours of colonies on VRBG are shown in Table 2.18.

SCREENING AT ISOLATION

The suitability of VRBG supplemented with oxytetracycline as a primary isolation medium was tested alongside OGY and RBC for recovery of yeasts from 6 different meat samples (Table 2.19). Using a 2-way analysis of variance (Sokal and Rohlf, 1981) of samples against media, there was no significant media effect at a 5% significance level. Figure 2.4 shows VRGBO which had been surface-inoculated with a dilution of sausage homogenate. Five major yeast colony forms are evident.

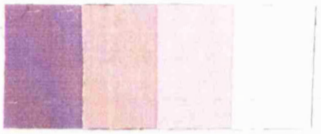
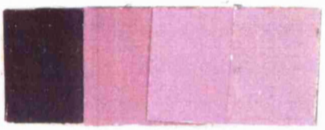
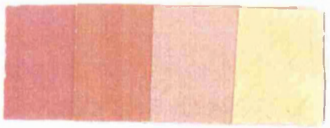
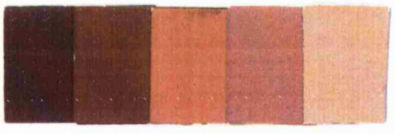
Table 2.17 Yeast growth and differentiation on a range of media

Media	Growth*	Differentiation	Comments
Cornmeal agar (Oxoid)	Poor	None	Mycelial growth enhanced, supports ascospore formation
Wort agar (Oxoid)	Good	None	Good for colony description
Potato dextrose agar (Oxoid)	Poor	None	
Eosin methylene blue agar (Lab M)	Good	Fair	Colour range limited
Violet red bile glucose agar (Lab M)	Good	Good	Colour range good, filamentous growth slow
Mannitol salts agar (Oxoid)	Poor	Poor	
MacConkey agar (Oxoid)	Poor	Poor	

* Growth after 5 d at 25°C

Figure 2.3. A VRBG plate streak-inoculated with 5 different yeast species. From the top; Candida zeylanoides, Debaryomyces hansenii, Cryptococcus albidus, Rhodotorula rubra and Candida lipolytica

Table 2.18 Macrocolonial colour patterns evident on Violent Red Bile Glucose Agar

Colour*	Genus
	*Candida*
	*Cryptococcus*
	*Debaromyces*
	*Pichia*
	*Rhodotorula*
	*Rhodosporidium*
	*Saccharomycopsis*
	*Sporidiobolus*
	*Sporobolomyces*
	*Trichosporon*

* Samples isolated from sausage and poultry were incubated at 25°C for 5 d.

Table 2.19 Suitability of VRBGO as a primary isolation medium

Meat sample[+]	Medium* OGY	RBC	VRBGO
a	$2.9 \times 10^6 \pm 1.05$	$2.1 \times 10^6 \pm 2.29$	$2.6 \times 10^6 \pm 1.41$
b	$2.6 \times 10^2 \pm 2.45$	$2.2 \times 10^2 \pm 3.45$	$2.3 \times 10^2 \pm 2.50$
c	$2.9 \times 10^2 \pm 3.47$	$3.1 \times 10^2 \pm 4.08$	$2.1 \times 10^2 \pm 1.98$
d	$2.6 \times 10^4 \pm 1.49$	$3.9 \times 10^4 \pm 1.70$	$7.0 \times 10^3 \pm 7.29$
e	$2.8 \times 10^4 \pm 1.87$	$2.4 \times 10^4 \pm 2.81$	$5.2 \times 10^3 \pm 0.87$
f	$3.2 \times 10^6 \pm 2.47$	$3.6 \times 10^6 \pm 3.05$	$2.8 \times 10^6 \pm 1.79$

* Mean cfu g^{-1} (± standard error); 6 replicates; Incubation at 25°C for 5 d.
OGY = Oxytetracycline Glucose Yeast Extract Agar (Oxoid); RBC = Rose Bengal Chloramphenicol Agar (Lab M); VRBGO = Violet Red Bile Glucose Agar (Lab M) + 100 mg l^{-1} Oxytetracycline (Oxoid)

[+] a = sausage, b = chicken breast, c = minced beef (gas-packed), d and e = minced beef, f = skinless sausage.

Analysis using a 2-way ANOVA of samples against media showed there was no significant media effect at 5%.

Figure 2.4 A VRBGO plate spread-inoculated with a dilution of sausage homogenate and incubated at 25°C for 5 d.

DISCUSSION

Oxytetracycline glucose yeast extract agar (OGY) was used to isolate yeasts and, throughout the study, colony appearance after five to seven days incubation at 25°C was recorded. This particular medium was chosen because colonies were larger on antibiotic than on acidified medium (Figure 2.1). Moreover they were more easily described on OGY than on Rose Bengal chloramphenicol agar (RBC), on which the colonies tended to 'run'. All Petri dishes containing RBC had to be wrapped in aluminium foil in order to prevent the photodynamic, cytotoxic effect of the Rose Bengal dye (Banks *et al.*, 1985a).

Advances in yeast taxonomy are currently being made by a combination of molecular biology and biochemistry. The impact on species delimitation of these methods is clearly illustrated by the following two examples. Determination of DNA base composition and homology (Yarrow and Nakase, 1975; Price *et al.*, 1978), which distinguish between haploid and diploid species, has enabled the number of *Saccharomyces* spp. to be reduced from more than forty (Lodder, 1970) to only seven (Yarrow, 1984). Twenty of the former species are now recognized as biotypes of a single species and others were transferred to the genera *Torulaspora* and *Zygosaccharomyces*. Studies on the composition of cell walls (Von Arx and Weijman, 1979; Verona and Florenzano, 1980), have helped to resolve the heterogeneity of the genus *Candida* by distinguishing between those species of ascomycetous and those of basidiomycetous affinity. The

cell walls of the former contain mannan and glucan but little, if any, chitin, whereas the latter contain only chitin. The genus remains heterogenous, however, since some species contain xylose in addition to glucose and mannose and others contain fucose, rhamnose and/or galactose, but not xylose (Meyer et al., 1984). Eventually, such techniques will no doubt radically amend the existing classifications. As yet they are inappropriate for the ecological study of foods, where physiological attributes (e.g. growth at low water activity, osmotolerance) other than those used strictly in classification are of importance in studying the fate of yeasts in food under various environmental conditions (Mossel, 1971).

In Chapter 1 it was noted that, should yeasts emerge as important spoilage organisms of novel foods, then there would be problems simply because the average food microbiologist is unlikely to know much about these organisms. The present study was based on an anticipated need for an identification scheme catering for the relatively small number of yeasts associated with meat and meat products. Yeasts rarely cause spoilage of chilled meats exposed to ambient temperatures because they are unable to compete effectively with bacterial contaminants (Banks and Board, 1987). When elective factors such as gamma irradiation (Johannsen et al., 1984; Hughes and Patterson, 1988) and frozen storage (Winger and Lowry, 1983) impede bacterial growth, however, then yeasts flourish and are the major potential spoilage organisms. If the shelf-life of meat products is extended using the factors mentioned above, or indeed, should 'super-chilling' (-1 to -5°C) be introduced to allow extended storage of items at depots before distribution, then there

would be a need for an effective and rapid identification scheme, in order to establish standards and identify important depots of infection. Such a scheme must be simple to use, cost- and space-effective and offer reasonably reliable identification of a particular group of yeasts. If additionally it were linked to a selective/differential medium, then it would permit ecological surveys within factories and also analysis of microbial associations in foods. Such schemes have been devised, most notably for clinical yeasts where the two most commonly used kits are the API 20C yeast system and the Uni-Yeast-Tek (UYT) system (Fuchs and Dolan, 1982). These authors concluded that both systems were adequate for rapid, presumptive identification, but that morphological studies and observations were still essential for definitive identification. Indeed, further biochemical tests were often necessary as the UYT kit contained few carbohydrates and the API lacked tests for both urease and nitrate. They also suggested that some of the tests performed poorly beause of strain variation, were of little use in delimitation and ought to be replaced by more efficient tests. Indeed, the test response variation between strains, possibly as a result of habitat, may explain why the UYT and API systems are of little use for the identification of yeasts isolated from non-clinical specimens. For the identification of such yeasts, the API kit may be used as a screening process whereby patterns of responses are used to differentiate between isolates. As a source of carbohydrates, the API kit would be time-saving but impractical due to cost.

Literature from the last twenty years described in Chapter 1,

which extended a review compiled by Deák and Beuchat (1987), revealed that of the 500 recognized yeast species, 114 have been isolated from meats and meat products. Such isolates are mainly restricted to six genera: Candida, Debaryomyces, Rhodotorula, Cryptococcus, Trichosporon and Pichia (Dalton et al., 1984; Hsieh and Jay, 1984; Dillon, 1988). Of the 114 species, about 35 are of common occurrence and they are distributed in the following genera (numbers in parentheses); Candida (20), Debaryomyces (1), Rhodotorula (4), Cryptococcus (3), Trichosporon (2) and Pichia (5).

Identification of the commonly occurring species of yeast in meats was successfully achieved using the classical procedure (pp. 141) with multipoint inoculation. In terms of materials, time and space this technique proved to be inefficient for large scale surveys and, consequently, alternative schemes were sought.

COMPASS (Anon., 1986) was considered to offer a possible solution to problems encountered with classical identification because theoretically it diminishes the requirement for space and materials as well as reducing the time needed for identification. In this study, COMPASS compared unfavourably with the classical identification scheme. Indeed, only four out of ten isolates were identified with the correct names and even so, alternative names were listed in three cases (Table 2.9). The equal weighting given to the tests appeared to be the major disadvantage of the COMPASS scheme. For example, nitrate assimilation is normally characteristic of a yeast genus or of species within a genus; some carbon assimilation tests vary from time to time with the same strain. It was therefore misleading to allow such properties to have the same

impact on the eventual identification when so few tests were used. Indeed, numerical taxonomy of bacteria is based on every character being given the same weighting thus enabling the grouping of taxonomical units (referred to as 'operational taxonomic units', OTU's) into taxa. The taxa are defined on the basis of overall similarity and not by their response to predetermined characters (Sneath and Sokal, 1973; Sneath, 1985; Sokal, 1985). These authors advocate the use of a reasonable number of taxonomic characters (more than 60) to obtain a numerical classification which is 'stable', in that, a substantial increase in the number of tests does not alter the resulting taxonomic structure significantly. When large numbers of characters are measured, the estimate of similarity obeys what they describe as a principle of inertia; COMPASS used only 46 tests and therefore many of these were heavily weighted. Michener and Sokal (1957) concluded that there was no rational means of weighting characters and therefore in practice they must all be given equal weight. In fact, characters omitted from an analysis are effectively given zero weight and characters with many states carry a heavier weight in determining similarities than those with few states. Sneath and Sokal (1973) found that when many characters were used, the analysis of similarity was only slightly affected by weighting certain characters (unless the weighting was extreme).

It is because of the multiplicity of the chosen characters, that overall similarities are calculated by computer and are usually presented as matching coefficients - as with COMPASS, or as dendograms which express statistical estimates of the number of

differences and similarities between strains (Lapage et al., 1970, 1973; Gower, 1975; Sneath, 1978, 1979). Amongst others, the genera Serratia (Grimont et al., 1977) and Staphylococcus (Seager et al., 1986) have been delimited into species in this way.

Before COMPASS, attempts had been made to delimit yeasts using numerical analysis but with little success. Campbell (1972) studied the following genera; Saccharomyces, Kluyveromyces, Pichia and Hansenula. He separated the phenotypically dissimilar strains of Sacch. cerevisiae into 10 species, irrespective of the fact that such phenotypically dissimilar strains were capable of interbreeding (Yarrow, 1972). In 1973, Campbell grouped strains of P. wickerhamiii and P. rhodanensis together despite the fact that these heterothallic strains do not hybridize (Van der Walt, 1959). A further example of such misestimation is found in Jones' (1975) analysis of 26 yeast strains. It established that a strain of the ascomycetous species Lipomyces starkeyi was more closely related to a strain of the imperfect, basidiomycetous species Bullera alba than to two strains of the ascomycetous species Sacch. cerevisiae and Hanseniaspora valbyensis. Sneath (1962) suggested subdivision of taxa at 85% and 65% similarity coefficient; if 85% matching separates species then the 65% limit should separate genera, but as shown by Campbell (1972, 1973, 1975), this was obviously not the case for yeasts. By studying non-fermentative genera he found (1973) that in many cases the overlap was so great that no satisfactory division could be made. For example, Hansenula and Pichia overlapped so extensively that certain species of the two genera showed 80% relatedness. Indeed, it is only because the two

genera can be distinguished by the nitrate reaction that the species remain separate, an obvious contradiction of the rule of equal weight to all characters. This example of numerical analysis highlights inadequacies in the basic taxonomy of yeasts. Recently, Kurtzman (1985) discovered that Hansenula mrakii and Pichia sargentensis share 68% DNA base sequence homology. Although this degree of relatedness is at the lower limit of that presently accepted between strains of species, it is clearly much higher than that found between species of different genera (Kurtzman, 1984). Consequently, this author anticipates that use of the nitrate reaction for delimiting either species or genus of these yeasts will no longer be valid. In fact, it is likely that saturn-spored species presently assigned to these genera will be transferred to Williopsis and the remaining species of Hansenula will be placed in Pichia (Kurtzman, 1984).

The problems outlined above, which were apparent in 1974 (Campbell), are still present in the COMPASS system. This is mainly because, unlike bacterial taxonomy, the available information concerning yeasts is as yet insufficient and for this reason certain tests are given a misleading importance. As was exemplified above, the closeness of Hansenula and Pichia cannot be resolved without suitable weighting of nitrate assimilation as a test of fundamental importance. Unfortunately, once the contribution of nitrate assimilation to the analysis has been increased, the programme is less able to deal with genera in which nitrate separates species or is unimportant.

The tests used in COMPASS were poorly chosen, and as there were

only 46, inevitably, significant weighting occurred leading to spurious identifications. In one instance, Sporidiobolus salmonicolor a pigmented, ballistosporous yeast was incorrectly named Leucosporidium antarticum, a non-pigmented, non-ballistosporing yeast. In several cases isolates were identified with ascospore-forming or glucose-fermenting yeasts when the organism had formed neither ascospores nor produced fermentation. For example, Candida zeylanoides which was non-fermentative, was incorrectly identified with Zygosaccharomyces rouxii despite the fact that due to its fermentative ability, the latter yeast causes many problems in the food and beverage industry (see Chapter 1). Thus, COMPASS was of little use on ecological grounds because no account of yeast reactions in industrial situations was made. In summary, COMPASS was unsuccessful for the identification of meat yeasts. It would seem that many more tests need to be included to improve the likelihood of a correct score (Dr. R.R. Davenport, pers. comm.) in which event the scheme may no longer be time-saving.

An alternative to the equal weighting involved in numerical taxonomy was first used by Barnett (1971). He analysed the importance of tests used in classical identification (Lodder, 1970) and placed these in order of usefulness. These were the tests which delimited the yeasts most efficiently, varied least within the operational taxonomic units and were therefore highly weighted. Such weighting increased the ease with which taxa could be discriminated and rapidly lead to a 'generalised distance'. Sneath and Sokal (1973) considered that this technique was best used for

'special purpose' rather than for general taxonomic analysis. In agreement with this, although Barnett's (1971) analysis considered the whole range of yeasts, it was not ideal for the separation of a particular genus such as Saccharomyces into species, where differentiation was not necessarily reflected in the assimilation results. The first step towards 'special purpose' taxonomy was the identification of yeasts isolated from strawberries (Buhagiar and Barnett, 1971). They chose 68 specific names from the literature as representing most of the yeasts associated with fruits. The responses of these yeasts to 40 physiological tests were analysed according to Gower and Barnett (1971) and Barnett (1971) and a sequential key was constructed involving 23 of the tests. Such a key was described for all yeasts recognized to date by Deák (1986), who further simplified the key by applying the same techniques to yeasts occurring in a restricted habitat, in this case food (Deák and Beuchat, 1987).

The simplified identification key devised in the present study, which was based on an extension of the literature survey done by Deák and Beuchat (1987), was used successfully for the naming of over 300 isolates from meat and poultry. Only twelve physiological tests together with simple morphological observations were required. Results were easy to read and difficult to misinterpret. A satisfactory identification was available within three weeks, compared with two to three months with traditional methods. By restricting the number of yeasts to those in a specific group and choosing only the most efficient tests, this procedure could easily be adapted for isolates from commodities other than meats.

For ecological surveys of the type undertaken in this work, where large numbers of Petri dishes led to vast numbers of isolations, it was imperative to describe fully a colony at the time of isolation, particularly as many yeasts lost certain properties (e.g. ascospore formation) on subculture. It was noted that yeasts exhibiting the same appearance were not necessarily the same species and vice versa. When large numbers of Petri dishes were examined and, therefore the number of colonies too numerous for detailed study, three representative colonies were taken for subsequent identification using the simplified in conjunction with the ecological key. The latter, based on schemes devised by Davenport (1974,1980a,1981,1985a,b), which relate yeast characteristics to environmental characteristics, was useful for a rapid determination of the overall profile of a group of yeasts and for separation into smaller groups. Such keys have been designed for yeasts in beverages and also for those in yoghurts (Davenport, 1980c). By adapting these methods to yeasts isolated from meats, patterns allowing the particular yeasts to be recognized, were obtained.

Group 3 was the largest of the four main groups described after incubation for five days at 25°C. It contained smooth and/or rough colonies and all of the Candida spp. common to meats (Appendix A). As noted above, the genus Candida is heterogenous and advanced taxonomical procedures are being developed to resolve this problem. Within the scope of the ecological key, however, delimitation was possible to a certain extent. Group 3 comprised yeasts which formed smooth colonies, produced no mycelium and assimilated very few

compounds, and those which formed smooth-becoming-rough or indeed rough colonies, produced mycelium, utilised a wider range of compounds and tended to exhibit various cell forms. Even at this level such observations are an indication of evolutionary developments; used in conjunction with more advanced techniques such as assimilation patterns, long-chain fatty acid composition and DNA base homologies, yeast phylogeny is at present being resolved (Viljoen et al., 1988; Kock et al., 1988).

Separation into groups was rapid and efficient once the description of colony morphology had been standardised. By using a range of simple chemical and physical tests and, where possible, evaluating adapation to a particular environment (e.g. osmotolerance) then separation into genera was achieved.

The possible use of commercial media for screening purposes in large ecological surveys and for highlighting certain properties of the isolates (Davenport, 1980c) was investigated. Several media were assayed for their utility as screening media. Violet Red Bile Glucose Agar supplemented with oxytetracycline (VRBGO) as a selective and differential medium allowed all of the yeasts to be characterised reliably. It was found that yeasts from different genera exhibited a particular colour and characteristic texture, and, on the basis of these properties, the proportion of a particular yeast within a yeast population could be assessed immediately. Subcultures from representative colonies were then identified using the ecological key in conjunction with the simplified key. The range of colours produced by yeasts on VRGB suggested relationships between certain genera (Table 2.18). The

similarity between colours of Debaryomyces, Pichia and Candida was particularly obvious and perhaps not too remarkable as certain Debaryomyces spp. bear resemblance to Pichia spp. in terms of habitat and metabolism and many of the imperfect states of these yeasts are represented within the genus Candida. Also, the limited colour range of Rhodosporidium occurred within the range of colours exhibited by Cryptococcus. These genera may be related; Rhodosporidium is a pigmented basidiomycetous genus that contains inositol positive and negative species, and has members that produce anamorphs of the pigmented genus Rhodotorula which are always inositol negative. The anamorph genus Cryptococcus is delimited from Rhodotorula purely because it is inositol positive, and its members are usually cream-coloured but occasionally pigmented. Moreover, it contains species which exhibit basidiomycetous characteristics (Rodrigues de Miranda,1984b). Thus, pigmented, inositol positive Cryptococcus spp. could be anamorphs of Rhodosporidium.

The present study required an identification scheme which would allow rapid and reliable classification of 'meat yeasts', without the need for advanced techniques, normally unavailable in an industrial laboratory. By analysing the methods available for yeast taxonomy, the most appropriate way to achieve this was to compile a simplified key involving only a limited number of tests. An ecological key was also used together with a differential screening medium (VRBGO) so that the large number of isolates obtained as a result of surveys could be assigned to groups and genera before being classified. This procedure was used in Chapter 3 for

screening and identification of yeasts from skinless sausage, and in Chapter 4 to investigate the linear distribution of yeasts in poultry processing plants.

CHAPTER 3

YEASTS IN SKINLESS SAUSAGE

Skinless sausage, a derivative of British fresh sausage, is heat treated so that its surface is coated with coagulated protein. This ensures retention of sausage shape once the cellophane tube is removed. Both types of sausage are based on a common meat emulsion containing rusks. During the warmer months skinless sausage commonly spoils due to surface yeast growth. Although a common spoilage problem, it has not been studied in detail. The present study investigated contamination of the processing plant and yeast colonisation of the surface of the sausage.

INTRODUCTION

Skinless sausage, a derivative of British fresh sausage, is heat treated so that its surface is coated with coagulated protein. The British fresh sausage is contained within a casing, made either from the submucosa from the small intestines of sheep or pigs or formed from reprecipitated collagen.

COMPOSITION AND MANUFACTURE

Both types of sausage are prepared from comminuted meat, rusk, salted seasonings (which include the preservative sulphite, as well as antioxidants - vitamins C and K), and colouring (e.g. carmine red). The ingredients, proportions of which are legally defined (Table 3.1), are mixed with iced water. Typical skinless sausages contain (%); meat, 65, rusk, 12.5, seasonings, 2.5, and water, 20 (Leads, 1979). The sausage texture is determined mainly by the rusk (bread or more commonly biscuit crumbs) which absorbs much of the added water. Rusk also contributes to the aroma and flavour of the sausage. Salt, (0.8 - 1.2% (w/v) of the product), contributes to the formation of a pseudoemulsion through extraction of muscle proteins (Swift, 1965). The legally permitted preservative added in the form of sodium sulphite or sodium metabisulphite, must not exceed a concentration of 450 $\mu g\ SO_2\ g^{-1}$ sausage at the time of sale (Anon. 1962).

The first stage in manufacture comminutes the diced meat, iced water and other ingredients in a bowl chopper. The rapid rate of chopping and the cold temperature form what is commonly termed an

Table 3.1 Standard specifications for sausage

PORK AND BEEF		PORK	
Ingredient	% (w/v) sausage	Ingredient	% (w/v) sausage
Beef flank	20.4	Lean pork	26.4
Pork back fat	11.7	Pork back fat	22.0
Pork head meat	9.6	Pork belly meat	7.0
Processed rinds	2.5	Pork head meat	7.0
MRM*	1.7	Pasteurised rinds	4.0
Flour		Rusk	12.3
Rusk		Water/ice	19.3
Water/ice	54.1	Seasoning	1.7
Seasoning		Polyphosphate	0.3
Polyphosphate			
	100.0		100.0

*MRM = mechanically recovered meat.

'emulsion' because it is presumed that the particles of fat are coated with proteins which are solubilised by the salt during mixing. It is the extremely thorough mixing which gives the skinless sausage meat such an even consistency compared with the more heterogenous texture of the British fresh sausage. The emulsion (or bowl mix) is extruded under pressure into cellophane tubing and links are formed mechanically to produce individual sausages. Coagulation of the outer surface is achieved by treatment with hot water. Crates containing chains of the linked sausage are immersed in hot water (2 min 10 sec at 71°C), followed by rapid cooling - first in a pre-chilling tank (2 min at 10-15°C), and then in a tank containing iced, chlorinated water (15 min at 0-4°C). Heating ensures that the sausage retains its shape once the casing is removed. This is done mechanically by a 'machine-gun' which slits the casing longitudinally so releasing the sausages which are then hand-packed, 8 into a plastic box. The boxes are wrapped in polyethylene and held in chilled storage (4°C) at the factory for 18-24 h before dispatch to depots and shops.

Skinless sausage was chosen for this study because, (1) yeasts are known to be the dominant spoilage organisms, and (2) it is the only sulphited-meat product which undergoes a heat treatment during processing.

Over the last twenty-five years, the microbial associations and effect of sulphite in British fresh sausage have been studied extensively (Dyett and Shelley, 1966; Dowdell and Board, 1968, 1971; Brown, 1977; Abbiss,1978; Leads, 1979; Banks, 1983; Dalton, 1984). In contrast, work done on skinless sausages has been of an

exploratory nature only (Hockley, 1980; Legan, 1981; Fielder, 1983). A detailed study of yeasts in British fresh sausage was done by Dalton (1984) but, although these organisms are commonly known to spoil the skinless sausage, as yet no detail about the types of yeast, or of how the heat treatment of this product may affect the role yeasts play in the microbial associations has been recorded.

MICROBIOLOGY OF STORED MEAT

Traditionally yeasts have played a minor role in meat spoilage, indeed Walker and Ayres (1970) concluded that yeasts were unlikely to cause food spoilage unless bacterial competition was diminished. This is because yeasts do not compete effectively with and are thus quickly outnumbered by bacteria capable of rapid uptake of easily assimilable nutrients (see Chapter 1). It seems that yeasts only cause spoilage when bacterial growth is impeded in some way so that the ratio of yeast to bacteria is approximately one to one (i.e. yeast:bacteria $\geq$ 1:1). For example, the addition of antibiotics to poultry was found to increase the proportion of yeast recovered from the carcass (Njoku-Obi *et al.*, 1957; Wells and Stadelman, 1958; Barnes and Shrimpton, 1958; Walker and Ayres, 1959); frozen storage (-5°C) of poultry, lamb and mutton resulted in an initial decrease of microorganisms and a subsequent increase in yeasts (Haines, 1931; Lea, 1931; Schmidt-Lorenz and Gütschmidt, 1969; Schmidt-Lorenz, 1982; Winger and Lowry, 1983; Lowry, 1984); radurisation appeared to select for psychrotrophic yeasts rather than the typical bacterial spoilage flora in beef (Ingram, 1975; Niemand *et al.*, 1981; Johannsen *et al.*, 1984), in frankfurters

(Drake et al., 1958); in fish (Eklund et al., 1965, 1966) and in poultry (Hughes and Patterson, 1988). Meat products preserved by the addition of sulphite provide another situation where inhibition of bacterial growth may occur. Thus, Dyett and Shelley (1962, 1966) demonstrated that the presence of sulphite (400-500 $\mu g\ SO_2\ g^{-1}$) in the British fresh pork or beef sausage inhibited the growth of coliforms and other Gram-negative organisms, and suppressed the growth of bacteria for 1-2 d. At 30°C, they found that unidentified Gram-positive bacteria were the main spoilage organisms of sulphited sausage. Christian (1963) recorded a flora dominated by Gram-negative rods in minced beef stored at low temperature and the development of a putrid odour. When sulphite (500 $\mu g\ SO_2\ g^{-1}$) was added, the flora was dominated by Gram-positive rods, a sour odour developed and shelf-life was increased 2 to 3 fold. Gardner (1968) also observed a similar response with sulphited, vacuum-packed baconburgers; shelf-life was extended by 2 d at 22°C, 10 d at 10°C and 28 d at 5°C. Both Dyett and Shelley (1966) and Gardner (1968) observed that sulphite exerted an initial lethal effect on the microflora and that subsequent growth was retarded throughout storage when compared with unsulphited controls. This initial lethal effect may be due to the larger proportion of active preservative present at the outset; this decreases progressively throughout storage (Brown, 1977; Banks, 1983; Dalton, 1984). In the British fresh sausage, containing 450 $\mu g\ SO_2\ g^{-1}$, inhibition of coliforms and selection of a Gram-positive flora comprised of Brochothrix thermosphacta, yeasts and homofermentative Lactobacillus spp. has been observed in many studies (Dowdell and

Board, 1968, 1971; Hurst, 1972; Brown, 1977; Abbiss, 1978; Leads, 1979; Banks, 1983; Dalton, 1984; Banks *et al.*, 1985). According to Christian (1963), sulphite increases the lag phase and suppresses the growth of the dominant organisms at chill temperatures. Perhaps this may explain the above observations in that sulphite inhibition of the growth of Gram-negative bacteria removed the competition which would otherwise prevent successful colonisation by the slower-growing Gram-positive bacteria. When the initial contamination of the British fresh sausage was low (*ca.* 10^4 cfu g^{-1}) then Dowdell and Board (1968) and Abbiss (1978) noted that the microbial association was dominated by yeasts. Yet another example of yeast spoilage due to effective competition with bacteria when the ratio of the two populations was approximately the same. They also found that yeasts, as well as the other dominant organisms - *Brochothrix thermosphacta* and *Lactobacillus* spp., grew in sulphited media, whereas the minor members of the association - *Pseudomonas* spp. and coliforms, did not. Banks (1983) suggested that the importance of particular organisms in the microbial association of sulphited meat products was a reflection of their tolerance to the preservative. He found that certain yeasts - *Candida*, *Cryptococcus* and *Rhodotorula* spp. - grew in broth (pH 7) with a sulphite concentration of 750 µg SO_2 ml^{-1}. Some strains of *Brochothrix thermosphacta* were also able to tolerate this level, whereas others were inhibited at 450 µg SO_2 ml^{-1} and *Lactobacillus* spp. were inhibited by a sulphite concentration of 250-400 µg ml^{-1}. The minor members of the associations tolerated much lower levels of sulphite, for example, *Pseudomonas* spp. were inhibited at

160-330 μg ml^{-1} and coliforms at 50-270 μg ml^{-1}. Dillon (1988) noted a similar effect in minced lamb and lamb products where sulphite inhibited the growth of pseudomonads, the dominant Gram-negative spoilage bacteria, and Enterobacteriaceae. Consequently, the slow-growing yeasts and Gram-positive bacteria - Brochothrix thermosphacta and Lactobacillus spp. - proliferated. According to Hammond and Carr (1976), Nychas (1984) and Nychas et al. (1988), inhibition of pseudomonads is probably due to the interaction of sulphite with the enzyme glucose dehydrogenase, thus preventing gluconate formation from glucose. Also, as sulphite impedes regeneration of NAD^+, an excess of NADH inhibits citrate synthase activity in Enterobacteriaceae but not in Gram-positive bacteria (Banks, 1983).

In summary, the British fresh sausage provides an extremely unstable microenvironment in which sulphite has a strong elective action on the flora. At the pH obtaining in sausage (pH 5.8-6.8), most of the preservative will be present as bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) ions - termed 'free sulphite' (Taylor et al., 1986). This is in contrast to wines (pH 2.8-4.2), where molecular sulphur dioxide occurs (Hammond and Carr, 1976; King et al., 1981). At the beginning of storage when the amount of active or 'free' sulphite is highest, the slow-growing Gram-positive bacteria and yeasts are selected. The Gram-negative pseudomonads and Enterobacteriaceae remain quiescent until the concentration of free sulphite falls to a level which is no longer inhibitory, whereupon they will start to grow once more. If at the outset, however, contamination was such that the ratio of yeasts to bacteria was

approximately the same, then by the time the sulphite level has fallen sufficiently to allow growth of the Gram-negative organisms, yeasts will already have achieved the status of a major contaminant.

In the skinless sausage a similar flora developed due to the elective action of sulphite, such that the dominant organisms were Gram-positive Brochothrix thermosphacta, Lactobacillus spp. and yeasts (Hockley, 1980; Legan, 1981; Fielder, 1983). A pasteurisation effect due to the heat treatment caused a 10-100 fold decrease in microbial numbers in the coat compared with the core (Hockley, 1980). Legan (1981) suggested that this might account for the climax population of yeasts being higher in the coat than the core. In agreement with Dowdell and Board (1968), he proposed that abundant yeast growth occurred because of the low initial contamination of the coat, the greater availability of oxygen at the surface and also because the heat treatment, and subsequent protein denaturation, might lead to an accumulation of nutrients in the coat. Legan (1981) found that the initial level of sulphite in the skinless sausage was lower, and that subsequent loss was more rapid, than in the British fresh sausage. In fact, skinless sausages were effectively sulphite free when the climax was achieved. These populations were higher than in British fresh sausage and were dominated by yeast despite concentrations of free sulphite being low enough to allow growth of Gram-negative bacteria. Legan (1981) considered that the heat treatment was responsible for the rapid loss of sulphite and observed a consistently lower concentration of sulphite in the coat than the

core. As well as possibly selecting for the more resistant yeasts, the heat treatment has a destructive effect on the sausage as a microenvironment which may also contribute to the success of these organisms as the common spoilage organisms of skinless sausage.

YEASTS IN SAUSAGE

Yeast spoilage of sausage is manifested by a cream or yellow pasty slime and was first documented by Kühl (1910) who isolated a white yeast from 'slimy' sausage. Cesari (1919) and Cesari and Guilliermond (1920) isolated several yeasts from dried sausage as did Mrak and Bonar (1938) who inoculated sterile sausages with such isolates and obtained a dull surface slime. They concluded that the slime comprised a mixture of yeast and bacteria and identified the main yeast with Debaryomyces hansenii. Drake et al. (1958, 1959) also isolated yeasts of this genus most commonly from the surface slime of packaged frankfurters, the following species were also common contaminants; Candida lipolytica, C. zeylanoides, C. catenulata, C. famata and Trichosporon pullulans. Dyett and Shelley (1962, 1966) were the first to study sulphited sausage. They considered yeasts to be associated with the production of off-odours after storage at 22°C, despite the larger size of the bacterial population. After an extensive study of British fresh sausage, Dowdell and Board (1971) concluded that, although yeasts (unnamed) were not generally numerically dominant, they should be considered a major part of the microbial association in terms of biomass.

Ramirez and González (1972) isolated Candida iberica sp.nov. from Spanish sausages. In an extensive study of meat and meat

products, Aboukheir and Kilbertus (1974) obtained 1037 isolates, of which Candida melinii and Rhodotorula rubra were the most commonly encountered. Species were represented by the following genera: Saccharomyces, Debaryomyces, Hansenula, Candida, Cryptococcus and Rhodotorula. Živanović and Ristić (1974) isolated species belonging to the genera Candida, Rhodotorula, Hansenula and Pichia, in a survey of the dried 'tea sausage'. Szczepaniak et al. (1975) examined the yeasts on fresh pork-meat products called 'serwolatka'. They isolated eight genera of yeast (in order of descending frequency): Candida, Debaryomyces, Sporobolomyces, Rhodotorula, Brettanomyces, Cryptococcus and Trichosporon. Abbiss (1978) identified 50 isolates from sausage with species from the following genera: Candida, Cryptococcus, Rhodotorula and Trichosporon. Pathogenic yeast such as Candida parapsilosis and C. tropicalis were recovered from Bologna type sausage, salami sausage and ham (Staib et al., 1980). Comi and Cantoni (1980a,b) investigated the yeasts on dried sausages and found isolates from the same genera as above, Debaryomyces hansenii being the most commonly isolated. Banks (1983) identified 89 yeasts isolated from unsulphited and sulphited pork sausage and assigned 53% to Rhodotorula and 7% to Trichosporon. He surmised that the survey, albeit of a limited number of yeasts, had shown that sulphite did not significantly influence the composition of the yeast flora in sausage. As mentioned in the previous section, Banks (1983) had shown that, compared with the dominant bacteria, yeast isolates from sausage were more tolerant of sulphite. Previously, Brown (1977) noted that the loss of free sulphite was faster in yeast-

dominated sausages and attributed this to increased binding by agents of yeast origin. Although Banks (1983) observed production of sulphite-binding compounds from pure cultures of yeast in broth systems, he could not link the growth of yeasts in sausage with free sulphite depletion. Yeasts can metabolise glucose or maltose to acetaldehyde, pyruvate and α-ketoglutarate which are all strong sulphite-binding agents (Rankine and Pocock, 1969; Taylor *et al.*, 1986). Dillon (1988) noted that the concentration of acetaldehyde ($60\ \mu g\ g^{-1}$ meat) in lamb burgers after 6 d at 5°C accounted for 34% of the bound sulphite although altogether 96% was bound. Dalton (1984) concluded that acetaldehyde concentration in sausages was positively related ($r = 0.89$, $n = 26$) to the amount of bound sulphite and accounted for 40% (1°C) or 70% (15°C) of the latter. As only negligible concentrations of the other recognized binding compounds were recovered from British fresh sausage (Dalton, 1984), acetaldehyde appeared to be the most important - although not the only one - in sausage and lamb burgers.

Dalton (1984) completed the first thorough study whereby the types of yeast in sausage were identified and the most common were tested for ability to produce sulphite-binding compounds. The majority of the yeasts she isolated belonged to the following genera: *Candida*, *Debaryomyces*, *Pichia*, *Cryptococcus* and *Rhodotorula*. From the literature listed above and from the review of yeasts isolated from meats and meat products in Chapter 1, it can be seen that a narrow range of yeast genera occur on meat. Dalton (1984) found that although sulphite did not appear to influence the range of yeasts, it did seem to favour the growth of representatives of

certain genera. Sausage yeasts with an oxidative metabolism or weak fermentative abilities were resistant to sulphite at neutral pH. Debaryomyces hansenii, Candida zeylanoides, Pichia membranaefaciens and Candida famata were found to produce acetaldehyde, Cryptococcus albidus grew well in sulphited broth but did not produce acetaldehyde and the same was true for Rhodotorula rubra, although the growth rate was retarded. She concluded that this reflected the situation which occurs in sausage, where sulphite favours the growth of Deb. hansenii (the dominant yeast) and certain Candida spp. probably at the expense of Cryptococcus and Rhodotorula spp. Dalton also provided further evidence to suggest that yeasts are probably the only organisms that cause sulphite-binding in meat products. She noted a positive correlation between the size of the initial inoculum of Deb. hansenii and the amount of sulphite bound ($r = 0.98$ at 1°C, $r = 0.92$ at 15°C). In fact, this increase in sulphite binding associated with increased acetaldehyde concentration reflected the rate and extent of yeast growth in the sausages.

Dalton (1984) also investigated the flora of skinless sausages and concluded that the course of manufacture did not modify the composition of the flora although the incidence of the ascosporogenous Deb. hansenii, the most frequently isolated yeast in British fresh sausage, was reduced. This observation contrasted with the findings of Put and De Jong (1980) who studied yeast contamination of heat treated beverages and concluded that the heat resistance of ascospores was the reason for such yeasts being contaminants. Dalton suggested several reasons for the reduced

recovery of this yeast; the absence of ascospores at the time of heating (Put et al., 1977) and also the solute content, water activity and the pH of the environment (Juven et al., 1978; Graümlich and Stevenson, 1978). She concluded that the yeast flora of meat and meat products was only marginally affected by ingredients and processing systems.

The aim of the present study was to investigate thoroughly the yeast flora of the skinless sausage to determine why these organisms are commonly responsible for spoilage of this product. Isolates were identified using the schemes described in Chapter 2 and were added to the list of 'Yeasts in Meats' compiled in Chapter 1. The role of yeasts, compared with that of the bacterial components of the microbial association, was examined during storage. Finally, the elective action of sulphite and the effect, if any, of heat treatment on the microbial association were determined.

MATERIALS AND METHODS

Factory Survey

Five points along the skinless sausage processing line (Figure 3.1) were sampled during the manufacture of particular batches of sausage; meat (ca. 100 g) from the bowl chopper and the tubing machine was removed aseptically using sterile bags, sausages before and after the cellophane tube was removed and several packs of sausage, were placed in a coolbox containing ice packs before returning to the laboratory, whereupon they were sampled immediately. Packs of sausage to be examined subsequently were stored at 6°C in a fridge such that free circulation of air occurred between packs.

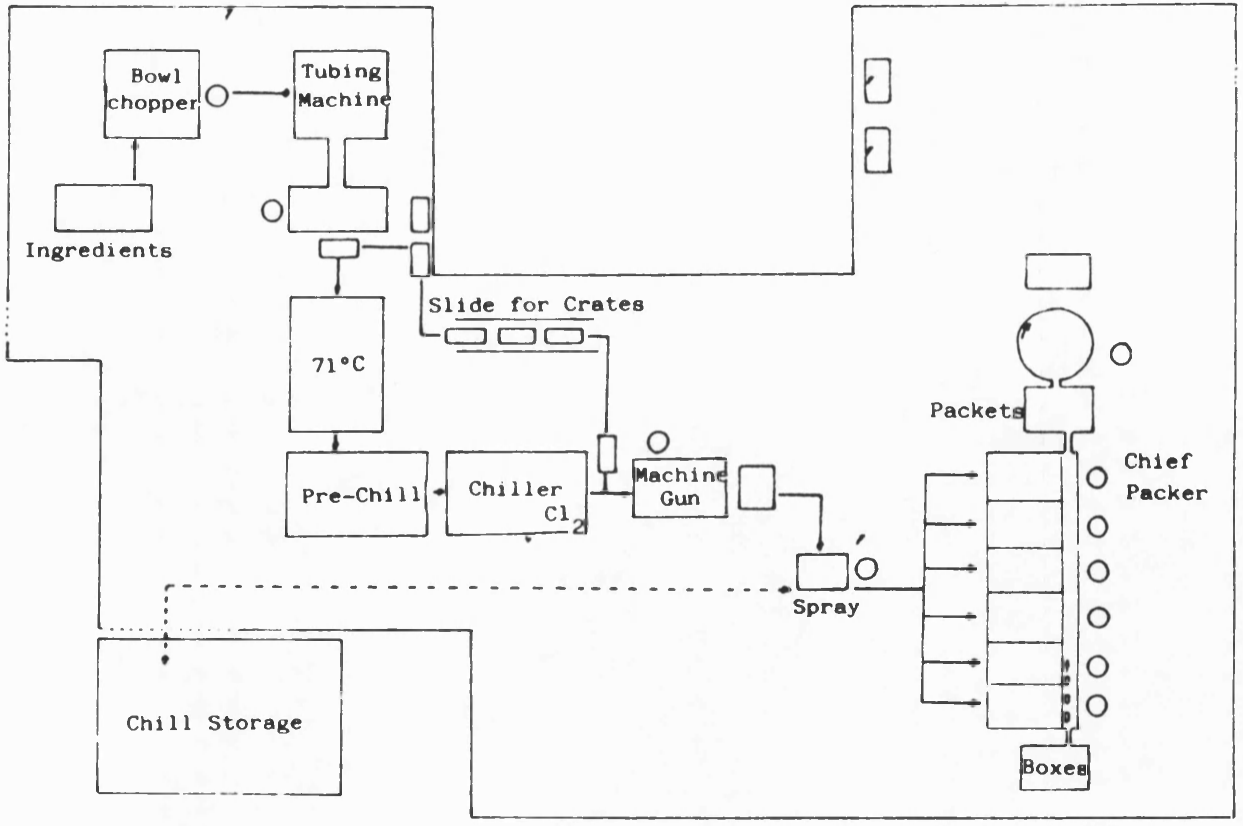
Chemical Analysis

The concentration of free, bound and total sulphite was determined using the method developed by Banks and Board (1982b).

Sample Preparation

A meat homogenate was prepared by vigorously shaking (for 30 sec through an arc of 0.5 m) a 5 g meat sample with 25 ml of chilled deoxygenated distilled water (flushed with nitrogen and kept on ice) in a screw cap bottle (100 ml) containing glass beads (4 mm diam.).

Figure 3.1 Plan of Skinless Sausage Processing Plant



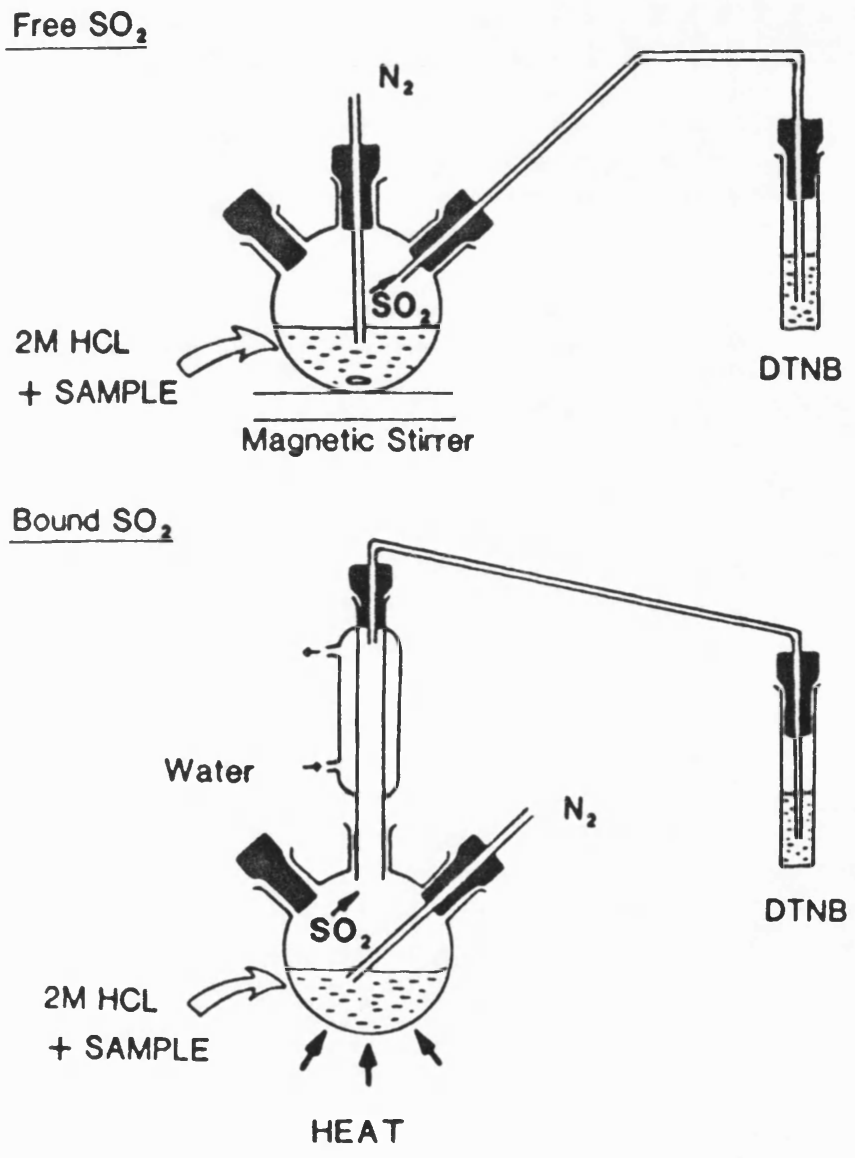
Free Sulphite Determination

The meat homogenate was immediately poured into a 250 ml, three-necked, round-bottomed flask (FR 250/322A - Scientific Glass Laboratories Ltd., Stoke-on-Trent, Staffordshire), containing a magnetic stirrer. Nitrogen was supplied (20 cm s^{-1}; Air Products, Bristol) *via* a modified Dreschel bottle head (24/29 SGL - with one of the side arms sealed by heating the glass), inserted into the centre neck (Figure 3.2). A second modified Dreschel bottle head was placed in a side neck and was connected to a third modified Dreschel bottle head with an elongated tip leading into a boiling tube (TT15/3). The tip was placed below the surface of a solution (25 ml) of 5,5-dithiobios 2-nitrobenzoic acid (2.3×10^{-3} M, DTNB) dissolved in phosphate buffer (pH 8) containing ethanol (10% w/v). Hydrochloric acid (20 ml, 2 M HCl) was added *via* the stoppered side neck. After 15 min the boiling tube was removed, phosphate buffer (25 ml) was added to the DTNB solution and the absorbance was measured at 412 nm (spectrophotometer SP6 550 UV/VIS, Pye Unicam). Sulphite concentration was determined after relating absorbance to a standard curve obtained by titrating iodine (0.01 N) against sulphite solutions containing starch granules.

Bound Sulphite Determination

The reaction vessel above was transferred to an electrothermal heater bed and connected *via* the centre neck (Figure 3.2) to a vertical condenser (C3/23 Quickfit, England). Nitrogen was supplied *via* a modified Dreschel bottle head in one side neck. A second modified Dreschel bottle head was placed at the top of the

Figure 3.2 Diagram of the apparatus used to determine free and bound sulphite



condenser and connected to a third modified Dreschel bottle head in a boiling tube with its elongated tip below the surface of the DTNB (25 ml) solution. The sample was heated for 15 min after which the boiling tube was removed, phosphate buffer (25 ml) added to the DTNB solution and the absorbance read at 412 nm.

Total Sulphite Determination

This was the sum of values obtained for concentration of free and bound sulphite.

pH Determination

The pH of the aqueous layer of the meat homogenate (10 g meat sample in 90 ml quarter-strength Ringer's solution, Oxoid) was determined using a pH meter (Pye Model 291, Pye Unicam).

Microbial Analysis

Enumeration of bacteria and yeasts in meat homogenates was followed by identification of the yeast isolates as described in Chapter 2.

Sampling of Meat

Ten grams of meat from the bowl chopper and the tubing machine were removed from the centre and edge of the sample with a sterile scalpel. Tubed sausages had their cellophane casing cut and carefully removed to avoid any water contaminating the surface. All sausages were dissected such that samples (10 g) of the core and

coat were transferred to sterile Petri dishes. The instruments used in handling samples were cleaned and sterilised between manipulations by first wiping on a paper towel, and then immersing in 100% (v/v) ethanol and flaming. Coat samples were obtained by removing strips of the outer layer (1 mm) of several sausages. Core samples were obtained from a single sausage by first removing the ends and the two sides to a depth of 5 mm and then transferring the sausage to a second sterile Petri dish and removing the remaining two sides (previously the top and bottom). Each sample was placed in a sterile stomacher bag with 90 ml sterile quarter-strength Ringer's solution (Oxoid) and homogenised for 60 sec in a Colworth Stomacher 400 (Seward, London). The homogenate was transferred to a sterile medical flat (200 ml) and allowed to settle into three layers. The top layer consisted of light meat and fat; the middle layer was a light pink, aqueous suspension of small particles, and the bottom layer consisted of the heavier meat and rusk particles. A range of serial dilutions (10 ml) of the middle layer were prepared in sterile diluent. The dilutions were shaken vigorously to ensure homogeneity and plated onto the various media described below. Three replicates of appropriate dilutions were set up on the selective media.

Enumeration of Microorganisms

The following media were used to enumerate the various microorganisms from sausage. Selectivity of each medium was tested by Gram-staining randomly selected colonies.

Total Viable Count

Samples (0.1 ml) of serial dilutions of sausage meat homogenates were spread on Plate Count Agar (PCA, Lab M) and incubated at 25°C for 3 d.

Enterobacteriaceae

One ml samples of serial dilutions of sausage meat homogenates were inoculated into 10 ml of molten (45°C) Violet Red Bile Glucose Agar (VRBG, Lab M). After setting a 10 ml overlay of molten medium was added. Incubation was at 30°C for 24 h.

Enterococci

One ml samples of serial dilutions of sausage meat homogenates were inoculated into 10 ml of molten (45°C) Kanamycin Aesculin Azide Agar (KAA, Oxoid). After setting a 10 ml overlay of molten medium was added. Incubation was at 37°C for 24 h.

Brochothrix thermosphacta

Samples (0.1 ml) of serial dilutions of sausage meat homogenates were spread on Streptomycin Thallous Acetate Actidione Agar (STAA, Gardner, 1966) and incubated at 20°C for 3 d.

Pseudomonads

Samples (0.1 ml) of sausage meat homogenates were spread on Pseudomonas Agar supplemented with 50 mg l^{-1} (w/v) centrimide-fusidic-acid-cephaloridine (Ps, Lab M) and incubated at 15°C for 3 d.

Lactobacilli

One ml samples of serial dilutions of sausage meat homogenates were inoculated into 10 ml of molten (45°C) de Man, Rugosa, Sharpe Agar (MRS, Oxoid). After setting a 10 ml overlay of molten medium was added. Incubation was at 25°C for 3 d.

Staphylococci

Samples (0.1 ml) of sausage meat homogenates were spread on Baird-Parker Medium (Oxoid) and incubated at 37°C for 24 h.

Yeasts

Samples (0.1 ml) of serial dilutions of sausage meat homogenates were spread on Oxytetracycline Glucose Yeast Extract Agar (OGY, Oxoid) and incubated at 25°C for 5 to 7 d.

Yeast Analysis

Identification of yeast isolates was carried out according to Chapter 2. The following techniques were used to study the five dominant yeasts; Candida zeylanoides, Debaryomyces hansenii, Cryptococcus albidus, Rhodotorula glutinis and Candida lipolytica.

Determination of Growth Rates

To the broth medium containing 0.3% (w/v) lab lemco powder (Oxoid) and 0.5% (w/v) mycological peptone (Lab M) was added 2% (w/v) glucose (AR grade, Fisons) sterilised separately at 10 psi flash in water. The modified lab lemco broth (LLB) was buffered

using sodium citrate phosphate to pH 6.0 (Dawson et al., 1969) and checked routinely with a pH meter (Pye Model 291, Pye Unicam).

A 0.2 ml sample was prepared from an 18 h (exponential phase) broth culture, inoculated into 50 ml of sterile LLB in an Erlenmeyer flask (250 ml) fitted with a gauze-covered cotton wool bung and incubated for 36 h in a shaking water bath (90 oscillations min^{-1}) at 25°C. Sterile water (0.1 ml) was added to the uninoculated control.

One ml samples were withdrawn immediately after inoculation and at every 3 hours until the culture was entering stationary phase. Cell density was determined by relating absorbance at 600 nm (SP6 550 UV/VIS, Pye Unicam) to dry cell weight.

Growth in Sulphited Broth

Five ml of freshly prepared sodium metabisulphite (Analar, BDH) solution was added to 45 ml of LLB to give a final concentration of 500 µg ml sulphite. Alternatively, 5 ml of sterile distilled water was added to the unsulphited control broths. Samples (0.2 ml) of pure yeast cultures were inoculated and determination of growth was again by relating absorbance at 600 nm to equivalent dry cell weight.

Dry Cell Weights

When absorbance of the broth cultures (above) approached A600 = 0.500, then samples; 2 ml, 1.5 ml, 1.0 ml and 0.5 ml, were placed into centrifuge tubes containing sterile water: 0 ml, 0.5 ml, 1.0 ml and 1.5 ml respectively, to form a series of dilutions. The

cultures were centrifuged at 2600 g for 20 min (MSE centrifuge) and washed twice with sterile water (10 ml) before further centrifugation. All of the liquid was decanted, sterile distilled water (9 ml) added and the tube vigorously shaken. Three ml of the suspension was placed in each of three Bijoux bottles, previously dried (24 h), weighed and covered with punctuated foil tops. These were dried to a constant weight (96 h) in a hot air oven at 103°C, cooled in a desiccator and weighed on an analytical balance. The mass of cells equivalent to an A600 of 1.000 was then calculated.

Determination of Cell Size

This was determined by examining a sample from the broth culture after 36 h with phase contrast microscopy (x400). The average length and breadth was calculated by measuring 20 cells using a calibrated eyepiece graticule.

Determination of Sulphite Binding

One ml samples were aseptically removed immediately after inoculation and every 12 h during the 36 h incubation period. The concentration of free, bound and total sulphite was determined by the method of Banks and Board (1982b).

Determination of Acetaldehyde

The concentration of acetaldehyde in the broth cultures was determined every 6 h using the Boehringer Mannheim assay kit, no. 668613.

Growth on Sodium Chloride Medium

Yeast cultures were streaked across the surface of a medium containing 0.5% (w/v) yeast extract (Lab M), 5% (w/v) glucose (Fisons), 1% (w/v) mycological peptone (Lab M), 3% (w/v) agar No. 2 (Lab M) and supplemented with 5, 10, 15, 20 or 25% (w/v) sodium chloride (BDH). After incubation at 25°C, cultures were described and examined microscopically (phase contrast x400) at 3, 5 and 7 d.

Determination of Lipolytic Activity

Twenty isolates, tentatively identified with Candida lipolytica according to their appearance on Violet Red Bile Glucose Agar (VRBG, Lab M) - see Chapter 2, were multipoint-inoculated onto Tributyrin Agar (Oxoid), incubated at 25°C and examined at 24 h intervals for the formation of a zone of clearing. This index of lipolytic activity was assessed by recording zones of < 5 mm as *, > or = 5 mm as ** and >6 mm as ***. Subsequently, isolates were identified according to Kreger-van Rij (1984).

Determination of Unusual Structures

Carotenoid Isolates - Ten isolates from the same skinless sausage sample were inoculated onto Gorodkowa Agar (Van der Walt and Yarrow, 1984) such that, in each Petri dish marked into 3 sectors, 2 pure isolates and a mixture of the two were represented. In this way each isolate was grown as a pure culture and also mixed with each of the other 9 isolates. Incubation was at 4°C with examination for conjugation and mycelium (Banno, 1967) every two weeks during a two month period.

Cryptococcus laurentii - Five isolates obtained from different batches of skinless sausage were identified with Cryptococcus laurentii (Rodrigues de Miranda, 1984b).The formation of hyphae and chlamydospores was determined in two stages; first by growing the yeasts on Yeast Morphology Agar (YMA, Difco) at 25°C for 48 h, then by mixing a loopful of a test pair, making 2 parallel streaks 3 cm apart on the surface of a Petri dish containing Malt Extract Agar (Wickerham, 1951) or Cornmeal Agar (CMA, Oxoid) or YMA and examining at weekly intervals during incubation at 15°C (Kurtzman, 1973).

RESULTS

Before concentrating on a detailed study of yeast growth on skinless sausages obtained from a manufacturer, a small survey was done on ten packets of the manufacturer's sausage obtained from shops as well as those from other manufacturers. The primary objective was to establish whether or not the material exhibited common features in respect of levels and types of contamination at spoilage.

Ten packets of a manufacturer's skinless pork and beef sausage bought over a period of one year were studied on the 'sell-by' date (7 d after manufacture) after storage at 6°C. The core and coat of the sausages were sampled separately for general bacterial and yeast counts (Table 3.2). Four of the packets obtained during late spring, summer and early autumn, exhibited signs of spoilage at the time of examination. The surface colour changed from pink to light pink with a grey-green tinge, a stale odour was evident and cream-yellow colonies eventually coalesced into a generalised slime. This was particularly noticeable in the cleft between sausages (Figure 3.3). The bacterial content of the core samples was higher than that on the coats with bacteria always outnumbering yeasts (Table 3.2). When yeast spoilage of the surface occurred, counts of these organisms in the core were at least eight-two times smaller than those of bacteria. It was concluded that growth in the core did not influence surface spoilage to any great extent. With the coat on the other hand, yeast counts were always larger than those in the core. Indeed, yeasts outnumbered bacteria in three of

Table 3.2 Contamination of skinless sausage at sell-by date[1]

Count[2]		Pack of Sausage[3]									
		1	2*	3*	4*	5*	6	7	8	9	10
Coat	Bacteria	6.21	6.96	5.49	5.93	5.45	3.69	6.05	5.16	6.97	5.23
	Yeast	5.53	6.34	5.54	6.51	5.60	2.96	5.23	4.24	6.26	4.08
Core	Bacteria	6.30	7.10	6.34	6.46	6.91	5.30	6.96	6.01	7.02	6.31
	Yeast	4.11	5.18	3.11	3.77	3.59	1.00	4.34	2.48	4.41	2.64

1. Sausages from the same manufacturer obtained from different shops.

2. Mean bacterial and mean yeast count expressed as $\log_{10}$ cfu g^{-1}

3. Skinless pork and beef sausage sampled from March 1986 - November 1986 at the sell-by date.

* Evidence of spoilage.

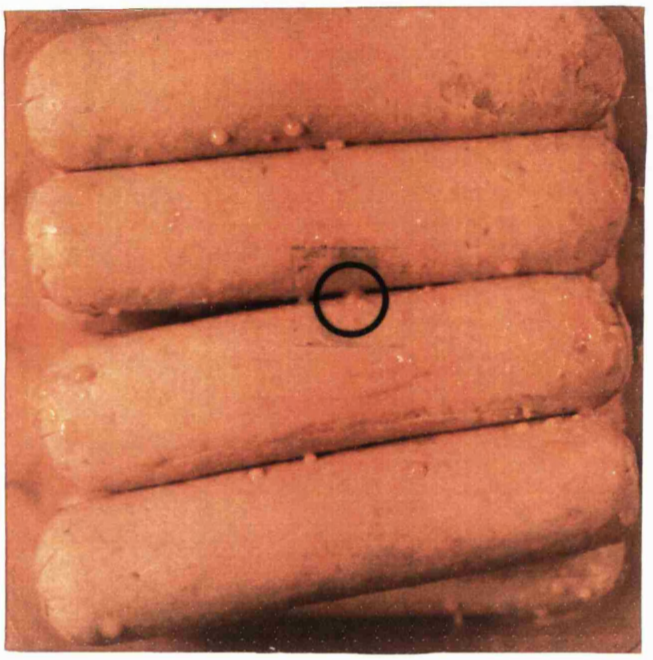


Figure 3.3 Skinless sausage manufactured in the summer showing spoilage (○) due to yeasts

the packets of spoilt sausage (Table 3.2). Even though the population of bacteria was three-fold larger than that of yeast in the fourth packet, the manifestation of spoilage (yellow slime) was still attributable to yeast.

According to Banks (1983) and Dalton (1984), sausage manufactured at the beginning of the week contained significantly higher microbial populations than that manufactured midweek. Banks (1983) attributed this to the use of meat which had been stored over the weekend and which therefore harboured larger numbers of microorganisms than that stored overnight. In the present work, contamination of ingredients was studied on four occasions during one year (Table 3.3). Sampling was always done on a Tuesday so that any seasonal trends could be detected. The stages in production of skinless sausage are given in Figure 3.1. Bacterial contamination was invariably greater than that of yeast at all stages of manufacture. In the raw ingredients (A) of Table 3.3 for example, the mean general bacterial contamination was 6.58 $\log_{10}$ cfu g^{-1}, with a maximum 7.88 $\log_{10}$ cfu g^{-1} in the summer. The mean yeast count was 4.20 $\log_{10}$ cfu g^{-1} and there was no obvious seasonal fluctuation. Swabs were taken of processing equipment, crates in which sausages were heated and stored, workers' hands, the rubber gloves worn at the time of packaging and samples of water used to spray the sausage before packaging were also taken (Figure 3.4). These revealed that processing equipment and crates were contaminated with the general flora of the sausage emulsion and that, in particular, the rubber gloves were a rich source of yeasts. Handling of the sausage emulsion coming from the bowl

Table 3.3 Contamination during processing of skinless sausages

Survey	Count[1]	Sampling Points[2] A	B	C	D	E
Winter	Bacterial	6.21	6.22	2.00	3.27	4.35
	Yeast	4.53	4.62	0.00	0.00	0.00
Spring	Bacterial	6.15	5.96	4.53	4.36	4.61
	Yeast	3.73	3.82	0.00	2.30	1.70
Summer	Bacterial	7.88	7.63	2.70	6.60	5.33
	Yeast	3.96	3.99	0.00	0.00	2.18
Autumn	Bacterial	6.08	5.99	3.92	4.75	4.25
	Yeast	4.57	4.85	0.00	2.70	2.00
$\bar{x}$	Bacterial	6.58	6.45	3.29	4.75	4.64
	Yeast	4.20	4.32	0.00	1.25	1.47

1. $\log_{10}$ mean cfu g^{-1} (6 replicate counts)

   $\bar{x}$ = mean untransformed counts

2. A = raw ingredients (bowl mix);

   B = mix before tubing;

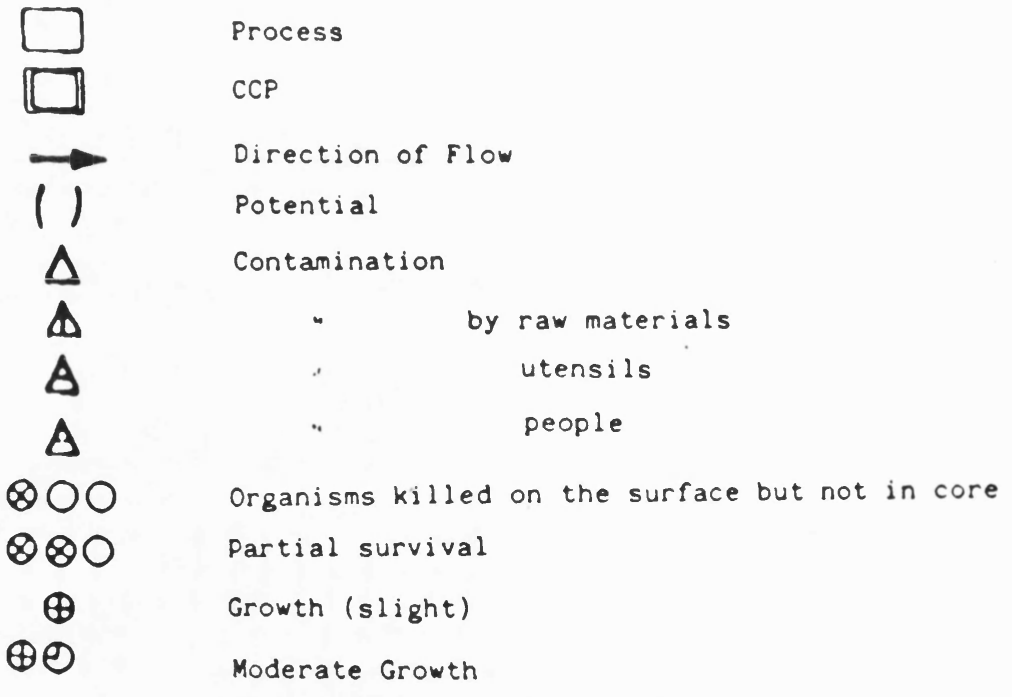
   C = coat after heating;

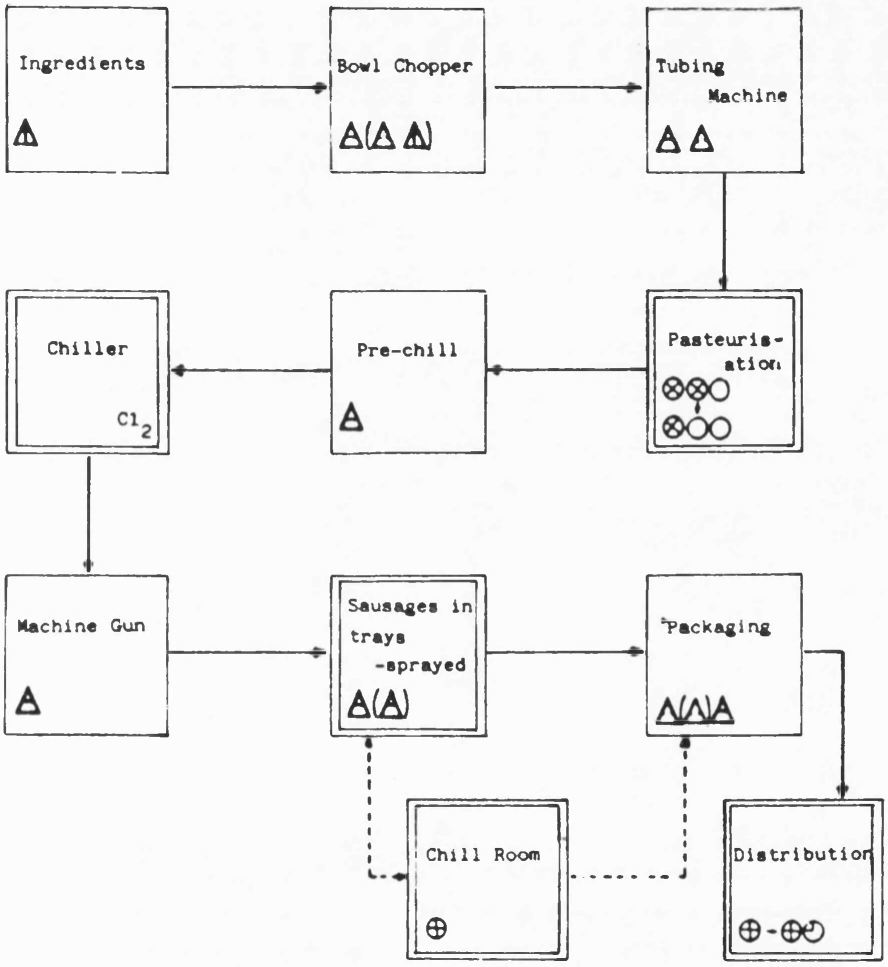
   D = coat after tube removal;

   E = coat after packaging.

   See Figure 3.1.

Figure 3.4 Flow diagram of skinless sausage production




Ingredients
Bowl Chopper
Tubing Machine
Chiller
Cl2
Pre-chill
Pasteurisation
Machine Gun
Sausages in trays -sprayed
Packaging
Chill Room
Distribution

chopper and contact with equipment led to a slight increase in contamination at the tubing stage (B in Table 3.3). Heat treatment (2 min at 71°C) of the sausage in the cellophane tubes had, as was to be expected, a dramatic pasteurising effect on surface counts (C in Table 3.3). Bacterial numbers were reduced by at least 96% and yeasts by an even greater extent. When the machine gun removed the cellophane tube from the sausage (D in Table 3.3), limited recontamination occurred as sausages were flung against the baffle plate of the machine and were subsequently stored in crates and sprayed with water before being packed (Figure 3.4). As noted above, further contamination occurred when the sausages (8) were packaged by women wearing rubber gloves. Thus, sausages ready for overnight cold storage on site prior to dispatch had a mean bacterial count of 4.64 $\log_{10}$ cfu g^{-1} and yeast 1.47 $\log_{10}$ cfu g^{-1} at the surface. It needs to be stressed that the extent of recontamination with yeasts in summer (x 150) and autumn (x 100) was greater than in spring (x 50) and winter (zero). General bacterial recontamination was greatest in summer (x 400) and winter (x 200) but negligible during spring (x 1) and autumn (x 2). The latter feature was investigated further by analysis of the range and types of bacterial contaminants (Figure 3.5).

MICROBIOLOGY OF SKINLESS SAUSAGE

Yeasts accounted for ca. 3% of the contamination of the emulsion formed from the raw ingredients (1 in Figure 3.5). Pseudomonas spp., Brochothrix thermosphacta, Enterobacteriaceae and lactic acid bacteria were the dominant bacterial contaminants;

Figure 3.5 Species compoisition during processing and storage (6°C)

as % of total flora

W = Winter

Sp = Spring

S = Summer

A = Autumn

1 = Raw ingredients*

2 = After heating

3 = After packaging

4 = After storage (6 d)

Y = Yeasts

P = Pseudomonads

B = Brochothrix thermosphacta

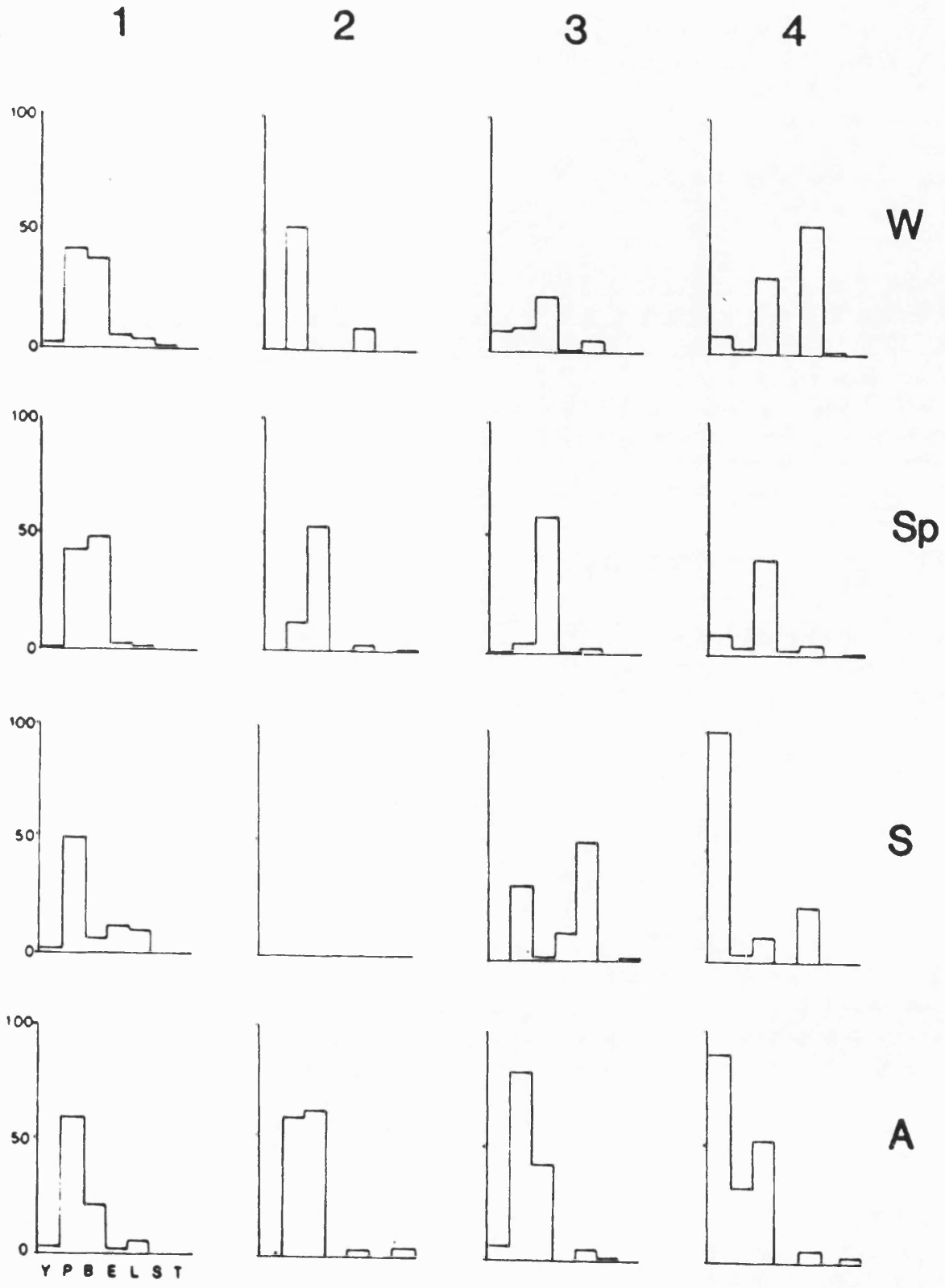
E = Enterobacteriaceae

L = Lactic acid bacteria

S = Staphylococci

T = Streptococci

* See Flow Diagram (Figure 3.4)


1
2
3
4
100
50
0
W
Sp
S
A
Y P B E L S T

Staphylococcus spp. and enterococci were also isolated in small numbers, i.e. less than 1% of the total population. After heat treatment (2 in Figure 3.5), yeasts were not isolated from the sausage coat but Pseudomonas spp., Brochothrix thermosphacta and, to a lesser extent lactic acid bacteria and enterococci were. Even so, the pasteurising effect on the surface resulted in the numbers of bacteria being reduced by more than 96%. The sausages were heated for longer than usual (> 10 min) in the summer, with the result that no organisms were recovered from the surface.

The organisms in the core were also affected, but to a lesser extent, by the heat treatment (Figure 3.6). Compared with the initial microbial load of the raw ingredients, the levels of contamination were 10-fold less in the core compared with 100-fold less on the coat. As far as bacteria were concerned, the pasteurisation effect persisted throughout storage for 7 d at 6°C in both the core and the coat of the sausage (Figure 3.6), bacterial growth being negligible during this time. Considerable yeast growth occurred in both sites during this period. Yeast counts in the core increased from ca. 10^2 to 10^6 cfu g^{-1} and on the coat from 10^1 to 10^5 cfu g^{-1}. After packaging (3 in Figure 3.5), the flora of a sausage's surface comprised mainly Pseudomonas spp., Brochothrix thermosphacta and lactic acid bacteria. There were relatively few yeasts and even less Enterobacteriaceae, Enterococci and Staphylococcus spp. As was noted above, yeast recontamination of the surface resulted form contact of the detubed sausage with equipment. Only the following yeasts acquired at this time led to spoilage; Candida zeylanoides, Debaryomyces hansenii, Cryptococcus

Figure 3.6 Changes in the levels of contamination of skinless sausage during storage at 6°C

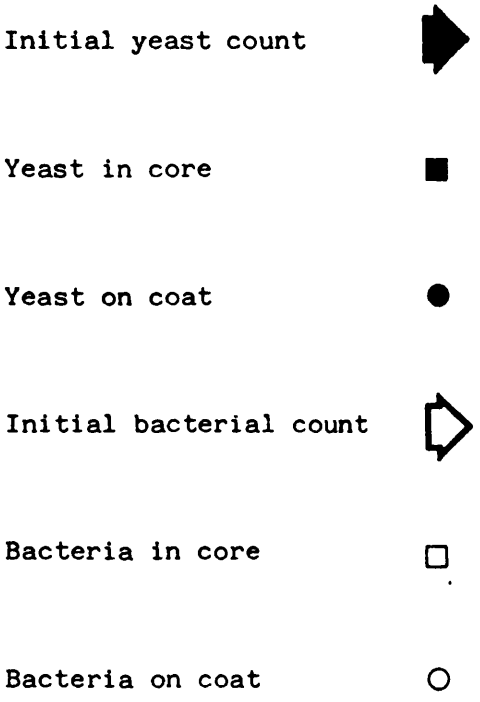
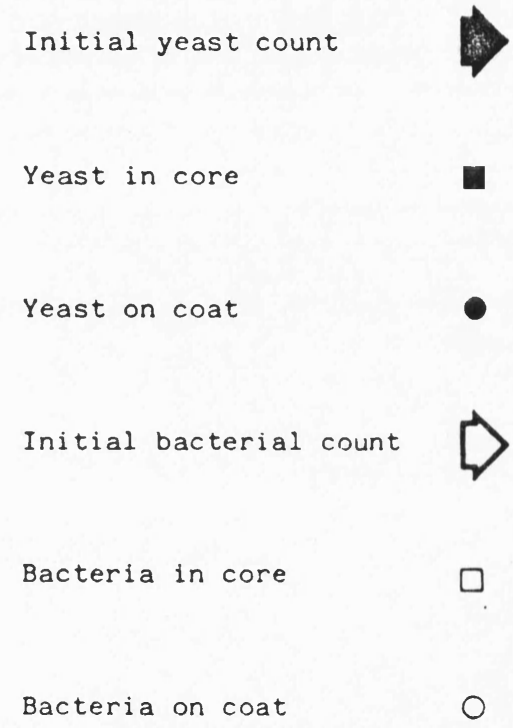
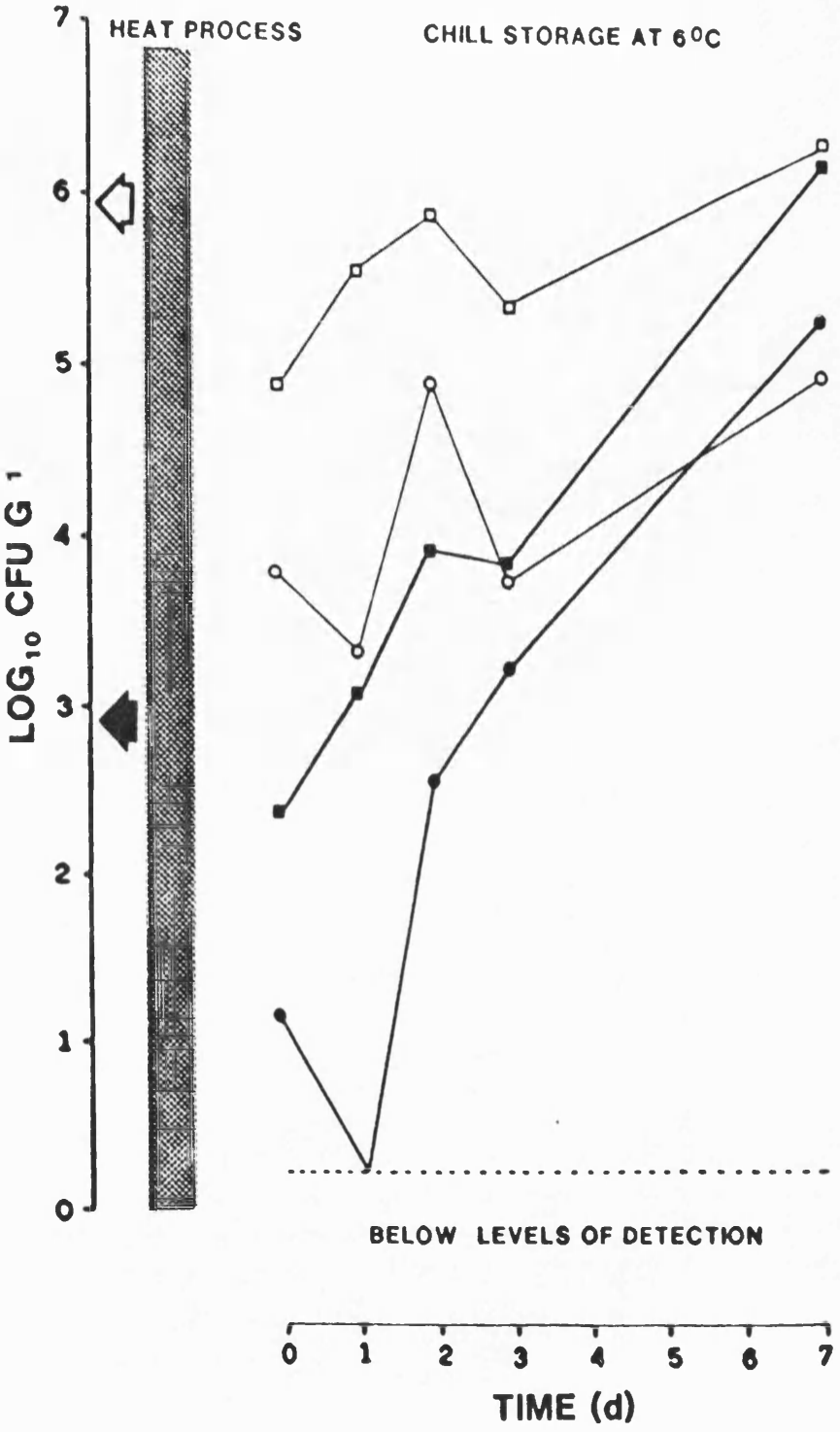


Figure 3.6 Changes in the levels of contamination of skinless sausage during storage at 6°C




HEAT PROCESS
CHILL STORAGE AT 6°C
LOG10 CFU G-1
7
6
5
4
3
2
1
0
BELOW LEVELS OF DETECTION
0 1 2 3 4 5 6 7
TIME (d)

spp., Rhodotorula spp. and Candida lipolytica. Adventitious yeasts including Trichosporon spp., Pichia carsonii, Sporidiobolus ruinenii and Candida tenuis were isolated at this time.

Packets (x 6) taken immediately following packaging, were stored for 7 d at 6°C until the 'sell-by' date, whereupon the coat was sampled (4 in Figure 3.5). A Gram-positive flora of Brochothrix thermosphacta, lactic acid bacteria and yeast had been selected by this time. In the autumn samplings, Pseudomonas spp. formed a substantial portion of the flora. This reflected the heavier recontamination of the surface at the time of packaging. Even so, only 50% of the pseudomonads survived storage. Indeed, the number of pseudomonads always diminished with storage. The numbers of Brochothrix thermosphacta always increased by ca. 10%. Yeasts and lactic acid bacteria were the only organisms to increase by more than 10%. In the winter samples, lactic acid bacteria increased by 50% and spoilage (souring) was associated with these organisms. In spring, they increased by 2% but the yeasts, which caused spoilage in some packets, by 10%. In the summer and autumn, the numbers of lactic acid bacteria increased by ca. 10% whereas those of yeasts increased by 90%. In this instance, yeasts were obviously the cause of spoilage (yellow slime).

In summary although recontamination of the surface with a diverse range of organisms occurred during packaging, a Gram-positive flora was selected during storage which was dominated by yeasts especially in the summer. This survey was followed by studies which attempted to identify factors favouring the growth of yeasts.

TYPES OF YEAST

In the course of this study 525 isolates were identified according to procedures described in Chapter 2. Of these, 336 were derived from the work described above, 63 from skinless sausage from other sources and 126 from various makes of British fresh sausage. Table 3.4 lists the yeasts identified in this study and in those of Banks (1983) and Dalton (1984). The colony morphology of yeasts isolated from various brands of skinless sausage and British fresh sausage is illustrated in Figure 3.7. It is noteworthy that all of the yeasts isolated in the present study have been previously associated with meats (see Chapters 1 and 2). The vast majority were assigned to six genera (% in parenthesis); Candida (45.9), Debaryomyces (29.1), Cryptococcus (11.8), Rhodotorula (6.5), Trichosporon (3.8) and Pichia (0.8). The other yeasts (2.1) were only isolated occasionally.

Five yeasts were commonly isolated from the surface of skinless sausage (numbers in parenthesis); Candida zeylanoides (110), Debaryomyces hansenii (85), Cryptococcus spp. (Cr. albidus - 21, Cr. laurentii - 17), Rhodotorula spp. (Rh. glutinis - 14, Rh. rubra - 18) and Candida lipolytica (35). The contribution of these yeasts to surface counts varied during storage, such that it was concluded that there was sequential colonisation. The sequence was Candida zeylanoides → Debaryomyces hansenii → Cryptococcus albidus → Rhodotorula glutinis → Candida lipolytica (Figure 3.8). This feature was particularly evident during the summer and autumn months when yeast growth was extensive (Figure 3.5). At the time of spoilage the dominant organisms were C. zeylanoides, Deb. hansenii

Table 3.4 Yeasts isolated from skinless and British fresh sausage

Genus Species	1*	2	3	4	5	6	7	8	9
Bullera									
alba								+	
tsugae								+	+
Candida									
boidinii	+								
cantarelli					+				
catenulata	+			+		+			
ciferii								+	+
curvata	+								
famata	+	+	+	+	+	+	+	+	+
humicola								+	+
ingens						+		+	
intermedia						+		+	
inositophila	+								
japonica	+								
lipolytica	+		+	+		+		+	+
magnoliae						+			
mesenterica								+	+
rugosa							+	+	+
sake								+	+
scottii	+							+	+
silvae								+	
steatolytica	+								
tenuis	+								
valida								+	+
vini					+			+	+
zeylanoides	+	+	+		+		+	+	+
Cryptococcus									
albidus	+		+	+	+	+		+	+
hungaricus									+
infirmo-miniatus	+								
laurentii	+	+						+	+
Debaryomyces									
hansenii	+	+		+		+	+	+	+
marama								+	
Hanseniaspora									
guilliermondii	+								
Hansenula									
polymorpha								+	
Pichia									
carsonii	+								
etchellsii	+							+	
fermentans					+				
membranaefaciens								+	+

Table 3.4 continued

Genus Species	1*	2	3	4	5	6	7	8	9
Rhodotorula									
glutinis	+	+					+	+	
minuta	+			+	+	+		+	
rubra	+						+	+	
Sporidiobolus									
ruinenii	+								
salmonicolor	+								
Sporobolomyces									
roseus	+								
salmonicolor	+								
Trichosporon									
cutaneum	+	+	+			+	+	+	
pullulans	+			+					

* 1, 2, 3 = skinless sausage

4, 5, 6 = British fresh sausage

7 = Banks (1983) sulphited British fresh sausage

8 = Dalton (1984) sulphited British fresh sausage

9 = Dalton (1984) skinless sausage.

Figure 3.7 Colony morphology of yeasts from various sausages

A Manufacturer (1): Pork and apple, British fresh sausage

B Manufacturer (1): Pork British fresh sausage

C Manufacturer (2): Pork and beef skinless sausage

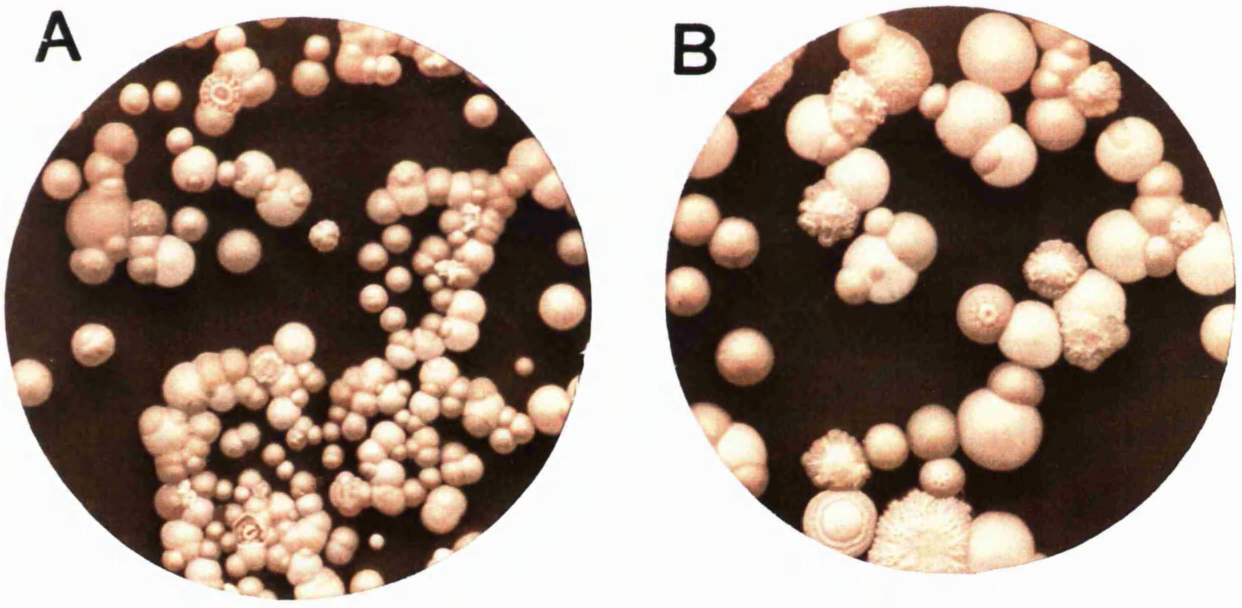
D Manufacturer (3): Pork and beef skinless sausage

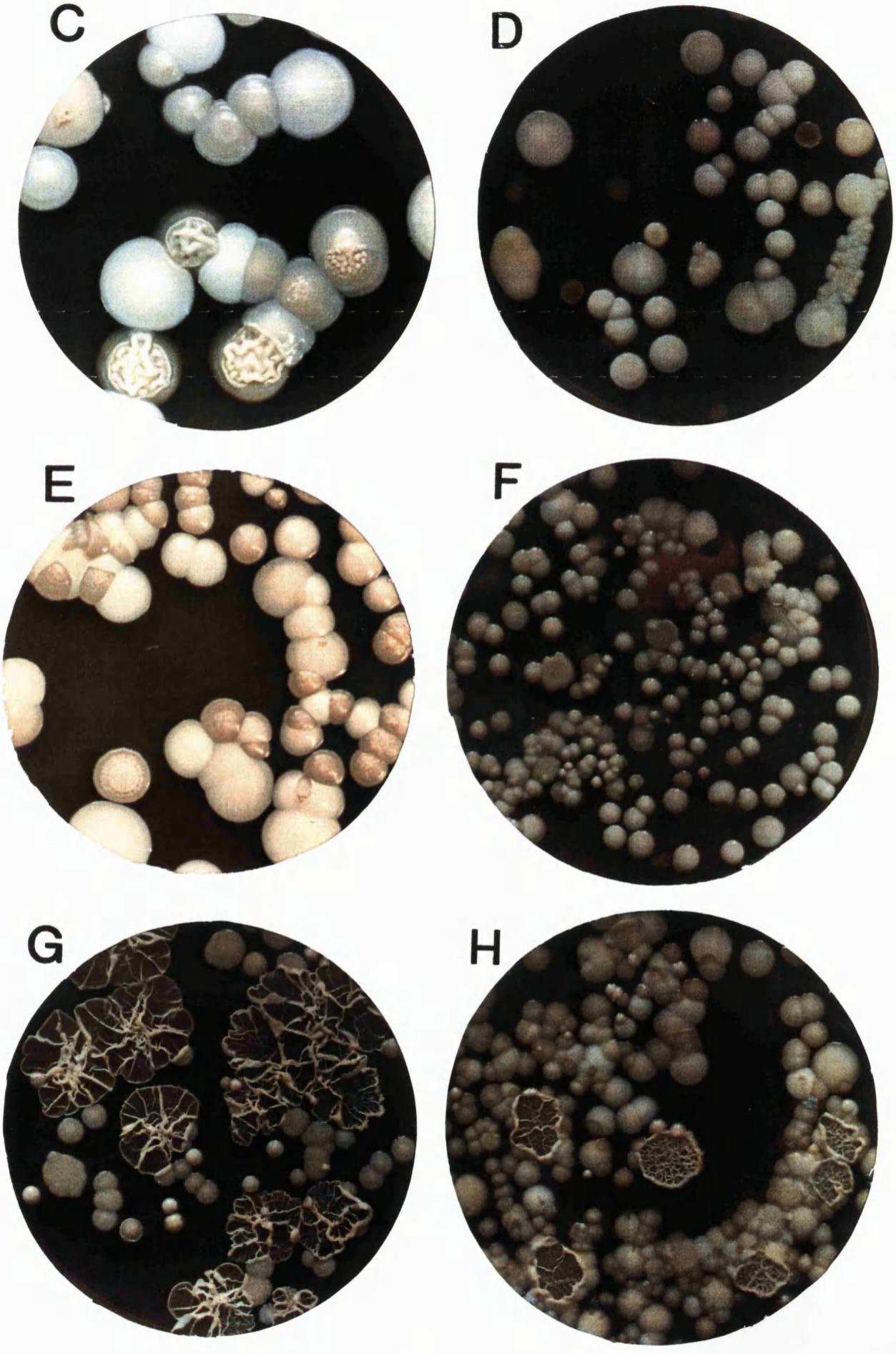
E Manufacturer (4): Pork skinless sausage

F Manufacturer (1): Beef British fresh sausage

G Manufacturer (5): Pork British fresh sausage

H Manufacturer (4): Pork British fresh sausage




C
D
E
F
G
H
10 mm

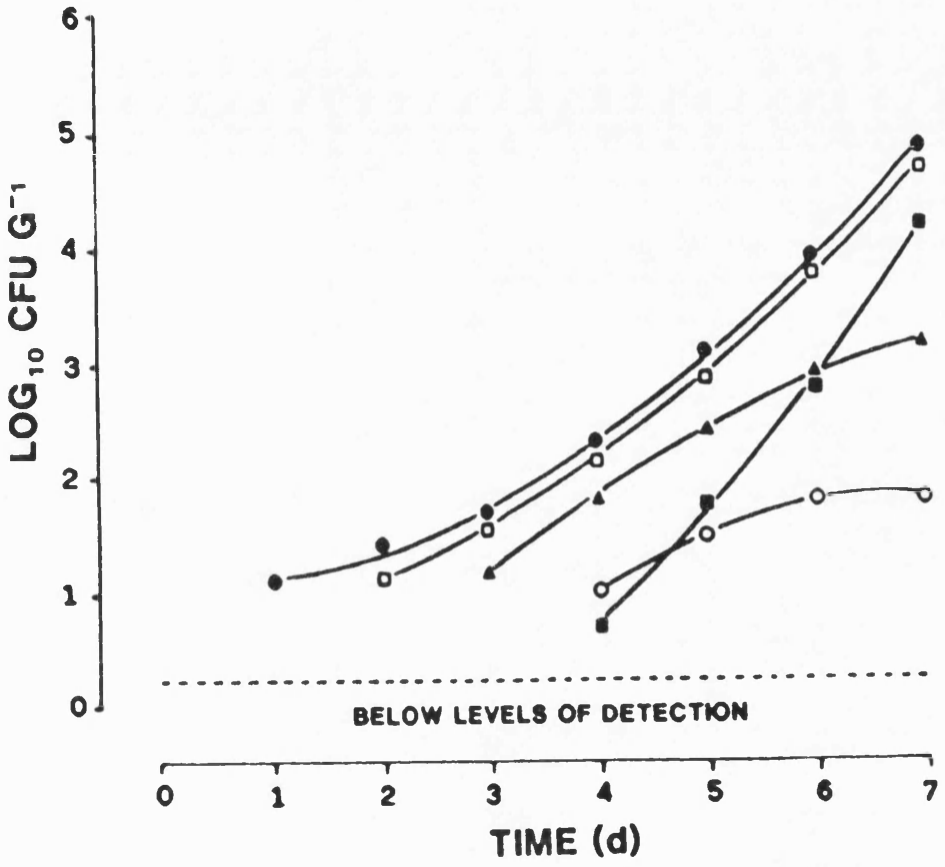


Figure 3.8 Colonisation of skinless sausage during storage at 6°C

● Candida zeylanoides

□ Debaryomyces hansenii

▲ Cryptococcus albidus

○ Rhodotorula glutinis

■ Candida lipolytica

C. lipolytica (Figure 3.9). In order to determine reasons for this specific order of recolonisation, the following characteristics of yeasts were investigated; growth rate, response to sulphite, physiological and morphological properties.

Rate of Growth

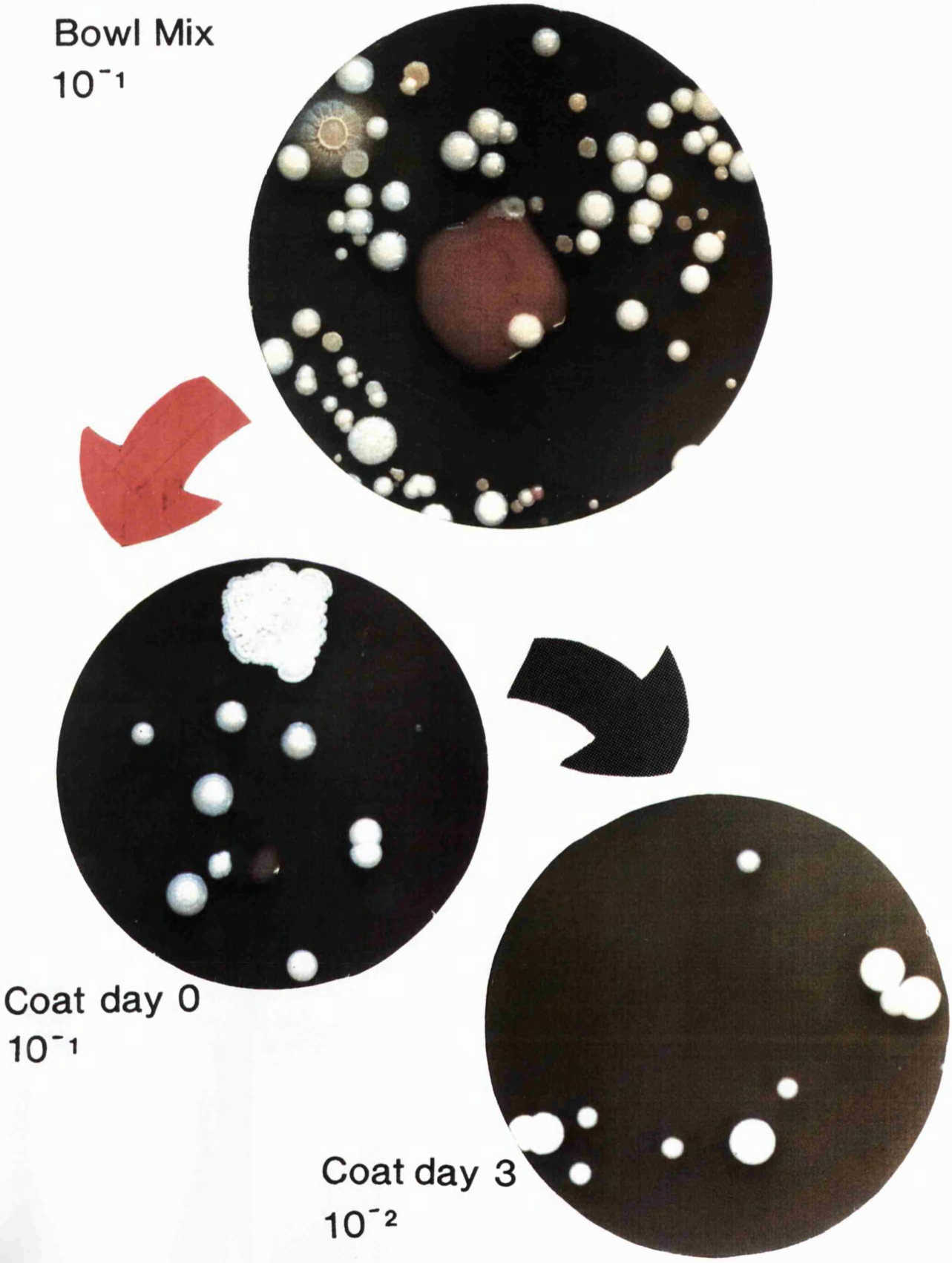
Throughout storage of sausages the pH varied little, the overall range being 5.8 to 6.6. For growth rate determination, representative yeasts were grown in a modified meat broth poised at pH 6.0 and incubated at 25°C in a shaking waterbath. The growth rates obtained in this study are given in parenthesis after the yeast name: Candida zeylanoides (1 h 3 min), Debaryomyces hansenii (2 h 12 min), Cryptococcus albidus (3 h), Rhodotorula glutinis (3 h) and Candida lipolytica (2 h).

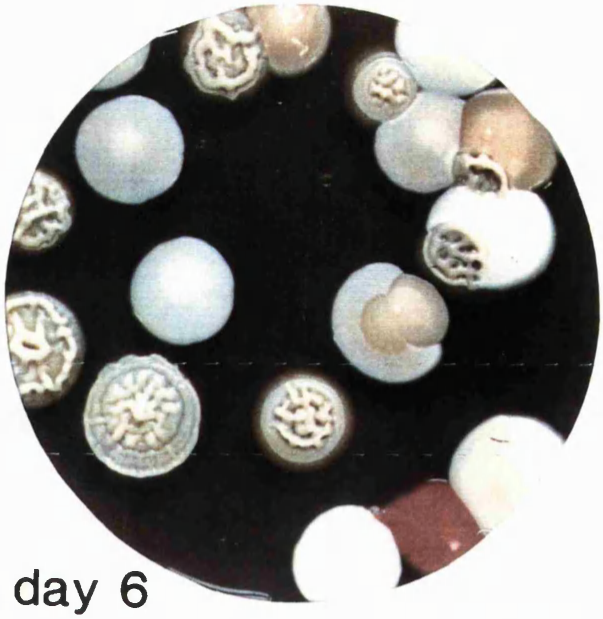
It was concluded that C. zeylanoides grew most rapidly (ca. 1 h), that both Deb. hansenii and C. lipolytica had similar growth rates (ca. 2 h) and that Cr. albidus and Rh. glutinis had the slowest growth rates (ca. 3 h). It should be noted that at the time of spoilage the dominant yeasts were those with the fastest generation times (C. zeylanoides, Deb. hansenii and C. lipolytica), and that although Cryptococcus and Rhodotorula spp. colonised the sausage surface before C. lipolytica, the latter organism rapidly outgrew them both.

Response to Sulphite

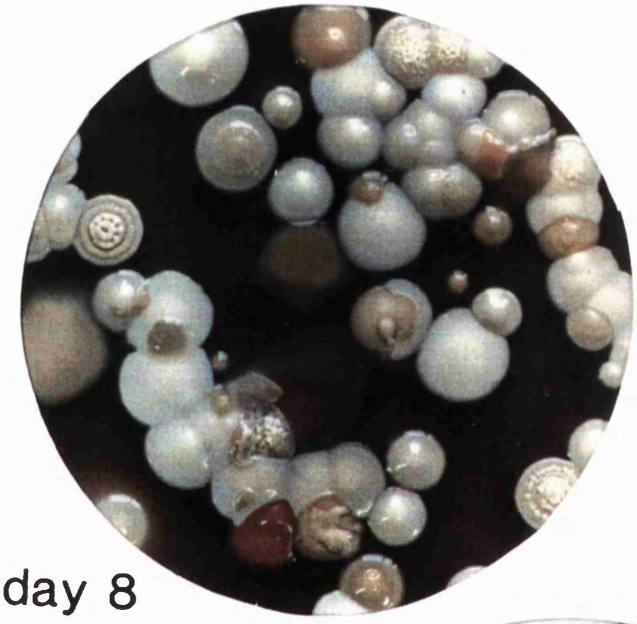
Tolerance of sulphite was considered to be another possible selective factor. Analysis of the sausage coat and core during

Figure 3.9 Colonisation of skinless sausage surface during storage at 6°C

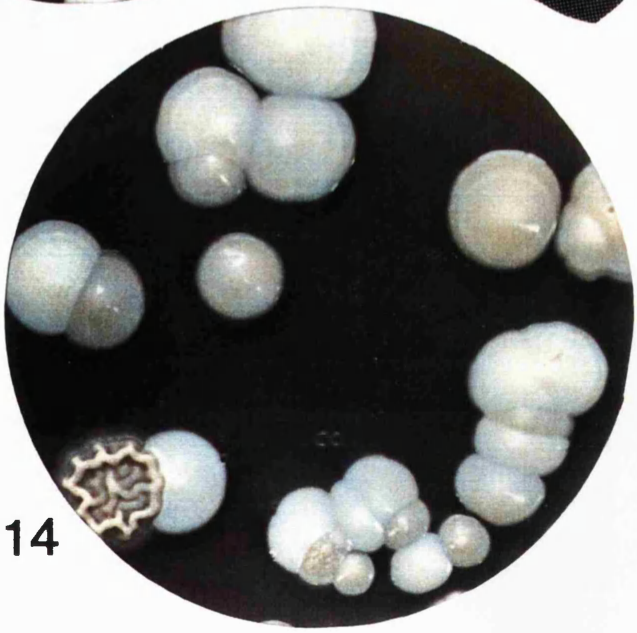




Coat day 6
10^{-3}



Coat day 8
10^{-3}



Coat day 14
10^{-6}

storage revealed a gradual loss of free sulphite (the active preservative moiety) and a concurrent increase in bound sulphite. The latter paralleled yeast growth at this time. It is evident from Figure 3.10 that none of the yeasts showed an exceptional response in terms of growth rates to sulphite (500 $\mu g\ ml^{-1}$), in a medium poised at pH 6. There were, however, marked differences in duration of the lag phase. Thus, Candida zeylanoides, Debaryomyces hansenii and Candida lipolytica had only slightly extended lag phases in sulphited broth compared with unsulphited control medium. Cryptococcus albidus and Rhodotorula glutinis had protracted lag phases (ca. 12 h). It is also evident from Figure 3.10 that some yeasts - C. zeylanoides, Deb. hansenii and Rh. glutinis - bound sulphite. Binding occurred during the exponential growth phase and was associated in two cases, C. zeylanoides and Deb. hansenii, with the accumulation of acetaldehyde.

Previous work has indicated that acetaldehyde is the major sulphite binding compound produced by yeasts (Brown, 1977; Banks et al., 1985b; Dalton, 1984; Dillon, 1988). Indeed, it is well established that this is also the case with wine and beverage yeasts (Burroughs and Sparks, 1964, 1973). The low pH's obtaining in such beverages cause an appreciable percentage of the free sulphite to be present as molecular SO_2 and this is bound before the yeast enters exponential growth (Warth, 1985). In this study both C. zeylanoides and Deb. hansenii produced acetaldehyde during the exponential phase of growth (Figure 3.10). An analysis of correlation of bound sulphite against the acetaldehde produced was significant ($p < 0.05$) for both C. zeylanoides ($r = 0.98$) and Deb.

Figure 3.10 Sulphite tolerance and binding ability from skinless sausage

A Candida zeylanoides

B Debaryomyces hansenii

C Cryptococcus albidus

D Rhodotorula glutinis

E Candida lipolytica

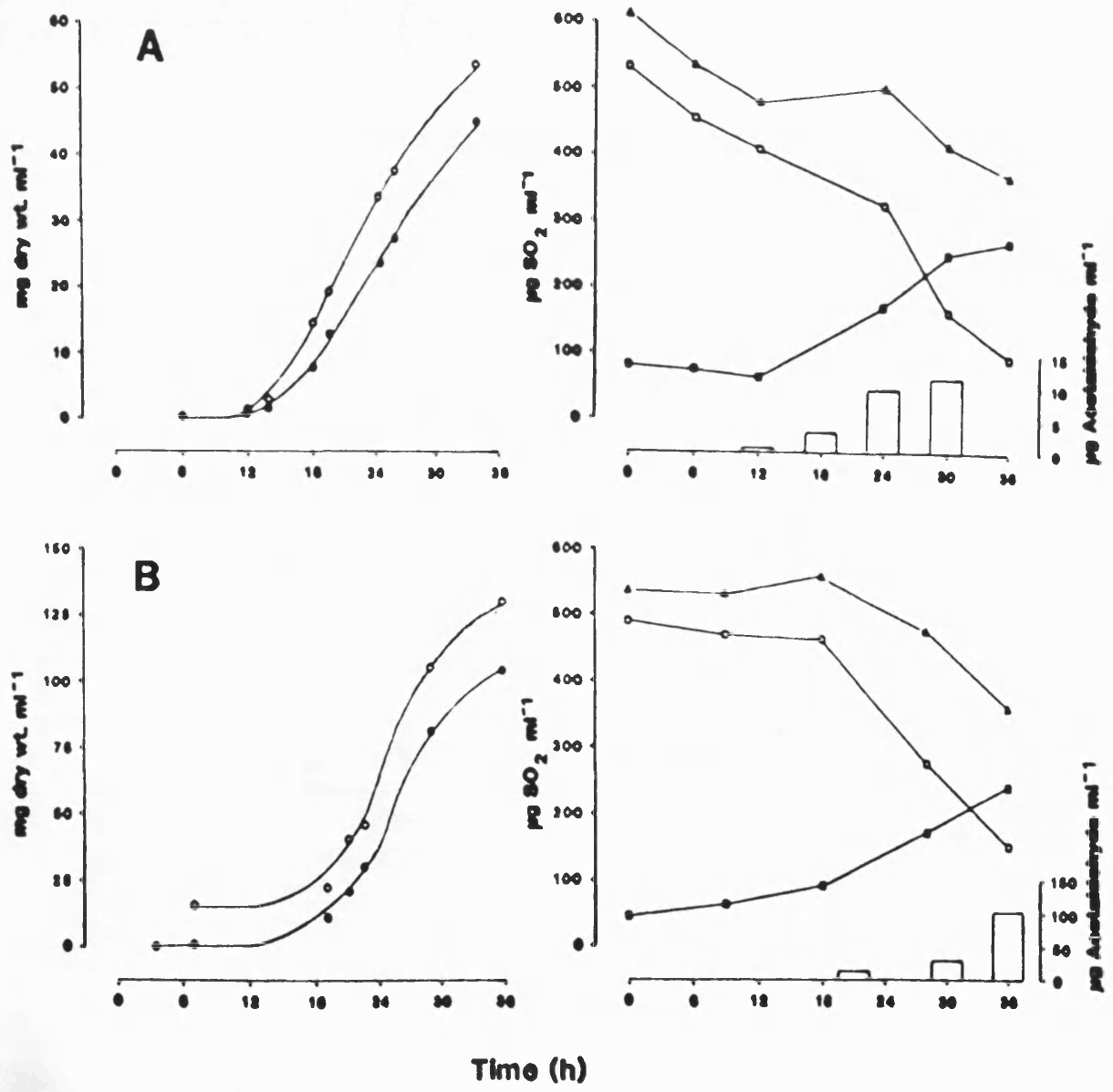
● Growth in sulphited broth (500 μg ml^{-1})

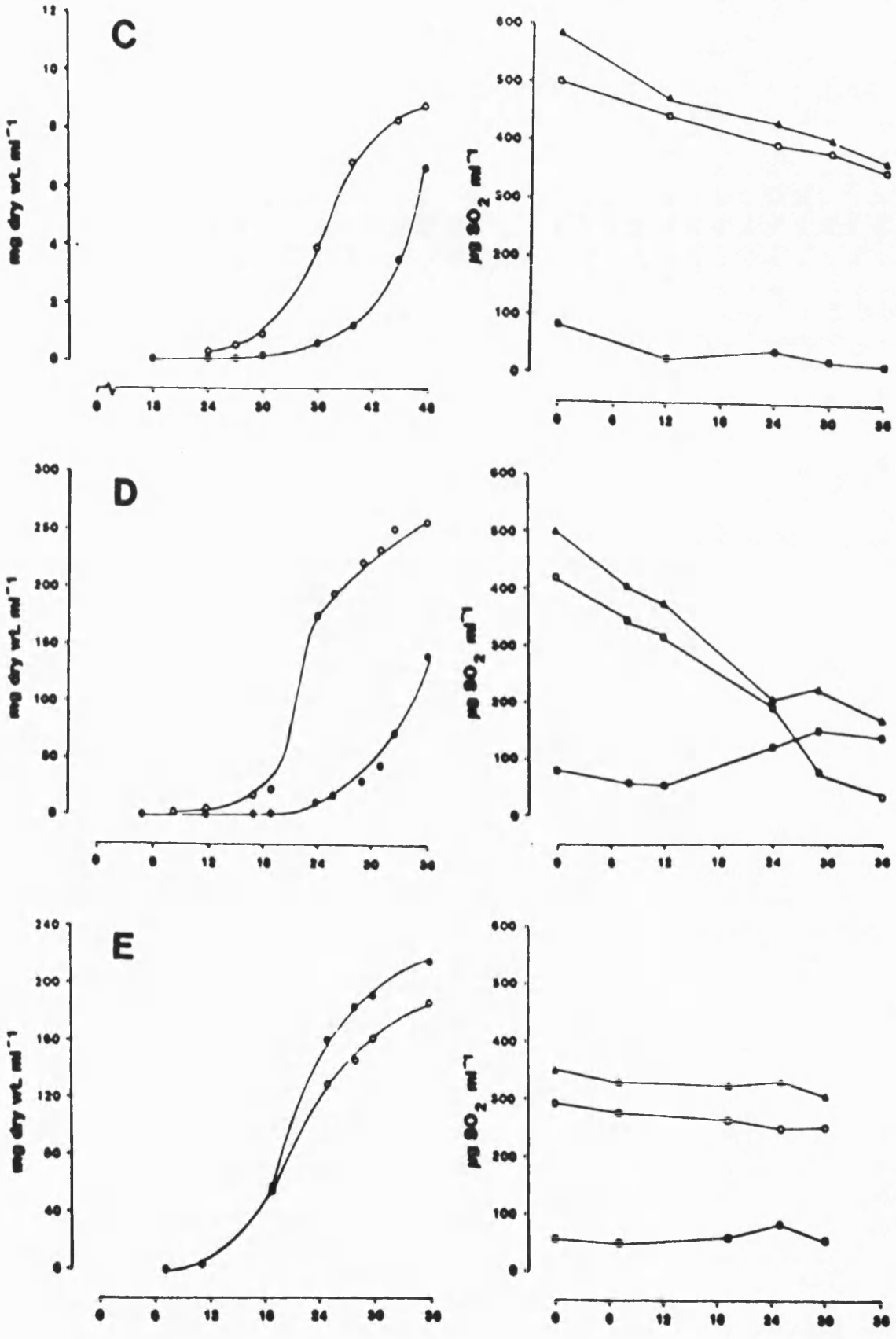
○ Growth in unsulphited broth

▲ Total sulphite

□ Free sulphite

■ Bound sulphite




C
D
E
mg dry wt. ml⁻¹
µl O₂ ml⁻¹ h⁻¹
Time (h)

hansenii (r = 0.93). Even so, as the acetaldehyde detected accounted for only 17.3% and 25.6% respectively of the total sulphite bound, other binding compounds must also have been present. In summary, sulphite did not appear to have a major elective effect on the order of colonisation of the sausage surface.

Physiological and Morphological Properties

Morphological characteristics, the ability to ferment glucose, assimilate a range of compounds and other physiological properties of the main yeasts in skinless sausage are detailed in Table 3.5. All of the organisms appeared well-suited to growth on this product for the reasons discussed below. The following properties (% of yeast isolates in parenthesis) are particularly relevant:

1. The sausage contains 0.8 - 1.2% (w/v) sodium chloride and yeasts (100%) were able to tolerate 10% (w/v) NaCl. The concentration of NaCl had a marked influence on colony morphology (Figure 3.11). Generally, an increase in concentration was accompanied by a restriction of growth and mycelium production; wrinkled or coiled colonies developed on media containing low salt concentrations but smooth colonies were characteristic of higher salt concentrations. Other yeasts produced large amounts of polysaccharide and formed flat 'spreading' colonies with low salt concentrations; an increase in NaCl caused a 'rounding-up' effect. Although of little importance in the sausage per se, water loss from meat debris on equipment could favour yeast rather than bacterial growth and therefore influence their

Table 3.5 Characteristics of the main yeasts isolated from skinless sausage

Test	YEAST 1	2	3	4	5	6	7
Morphology							
Pellicle	77	18.8	0	5.9	0	0	88.6
Pseudohyphae	100	30.6	0	0	0	0	100
True mycelium	0	0	0	0	0	0	100
Arthrospores	0	0	0	0	0	0	0
Ballistospores	0	0	0	0	0	0	0
Ascospores	0	81	0	0	0	0	0
Carotenoid pigment	0	0	0	0	100	100	0
Fermentation							
Glucose	4.5	7.1	0	0	0	0	0
Assimilation							
Glucose	100	100	100	100	100	100	100
Adonitol	100	100	100	100	100	100	0
D-arabinose	0	0	100	100	100	100	0
L-arabinose	0	0	100	100	0-	100	0
Arbutin	100	57.6	100	100	100	100	0
Cellobiose	0	11.8	100	100	100	16.7	0
Citric acid	0	0	71.4	82.4	42.9	27.8	100
m-Erythritol	0	21.2	0	100	0	0	100
Ethanol	59	100	23.8	11.7	100	100	100
Galactitol	0	0	0	100	0	0	0
D-galactose	80.9	100	47.6	100	100	100	41.7
Glucosamine-HCl	76.4	10.6	14.3	100	0	0	0
Gluconic acid	0	0	100	100	100	27.8	0
Glycerol	100	89.4	76.2	52.9	100	33.3	100

Table 3.5 continued

Test	YEAST						
	1	2	3	4	5	6	7
m-Inositol	0	0	100	100	0	0	0
Inulin	0	0	0	17.6	100	0	0
Lactic acid	0	17.7	0	0	0	0	91.4
Lactose	0	0	85.7	100	0	0	0
Maltose	0	100	100	100	100	100	5.7
Manitol	100	100	100	100	100	100	100
Mannose	100	100	100	100	100	100	100
Melibiose	0	57.6	9.5	100	0	0	0
Melezitose	0	100	71.4	100	100	100	0
α-M-glucoside	0	100	100	100	100	0	0
Raffinose	0	100	100	100	100	100	0
Rhamnose	0	45.9	90.5	100	100	100	0
Ribose	0	0	47.6	100	100	100	100
Salicin	57.3	100	100	100	100	100	0
Sorbitol	100	100	100	100	100	100	51.4
Sorbose	100	100	100	47.1	100	100	100
Starch	0	0	0	0	0	0	0
Succinic acid	50	100	80.9	100	100	100	100
Sucrose	22.7	100	100	100	100	100	14.3
Trehalose	100	100	100	100	100	100	0
Xylitol	0	84.7	80.9	100	100	100	22.9
Xylose	0	100	100	100	100	100	0
Ethylamine-HCl	100	100	66.7	0	100	100	100
Nitrate	0	0	100	0	100	0	0
Nitrite	10	28.2	85.7	35.3	100	0	0
Creatine	0	100	0	0	0	0	0

Table 3.5 continued

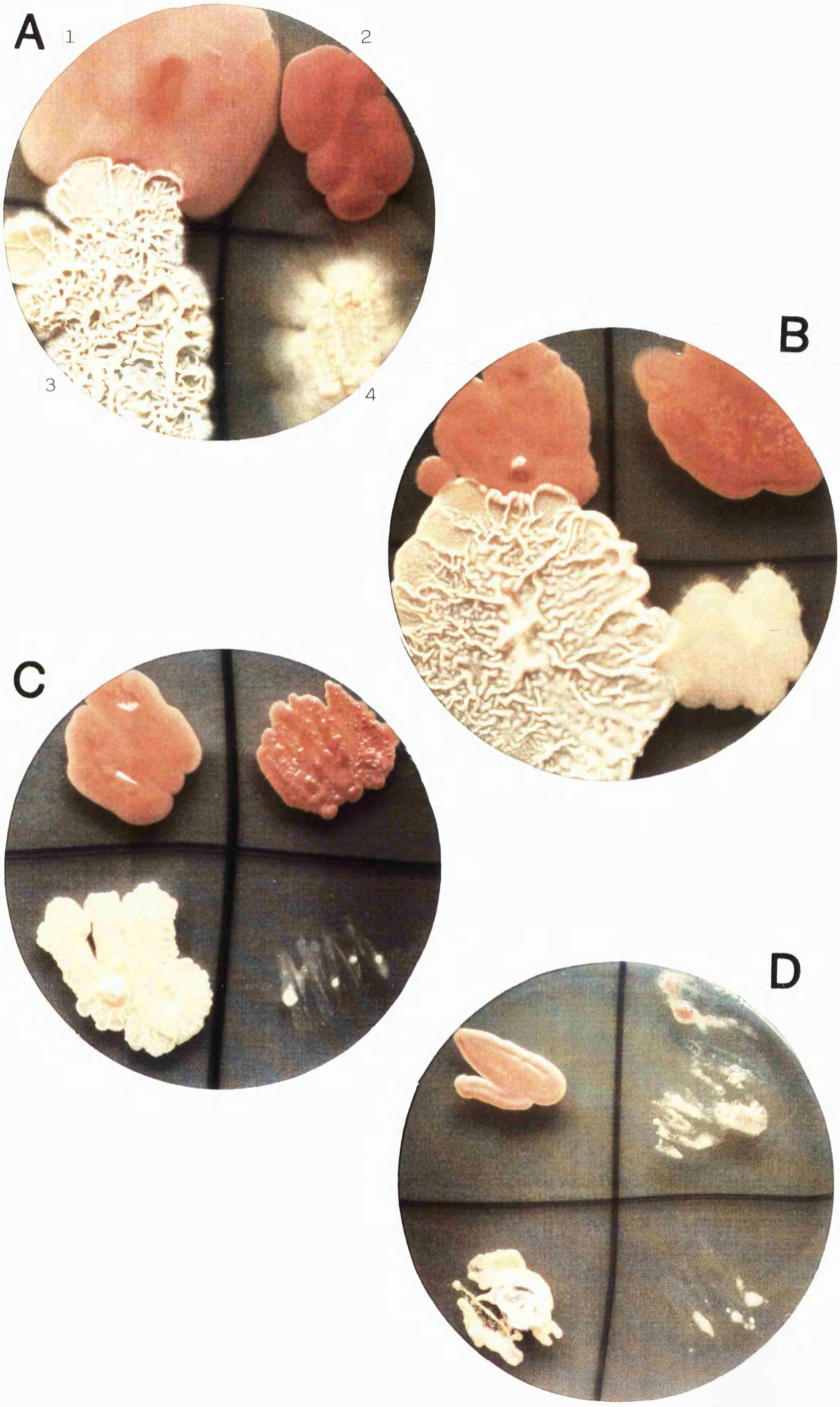
Test	YEAST 1	2	3	4	5	6	7
Growth							
Vitamin free	40.9	100	0	23.5	100	0	0
10% (w/v) NaCl	100	100	100	100	100	100	100
5°C	100	100	100	100	100	100	100
37°	0	0	0	17.6	0	0	5.7
Cycloheximide (w/v):							
0.01%	100	62.4	0	0	0	11.1	100
0.1%	100	48.2	0	11.8	0	0	100
Production							
Starch	0	0	100	100	0	0	0
Hydrolysis							
Urea	0	0	100	100	100	100	100
Arbutin	100	100	100	100	100	100	100
Gelatin	39.1	0	100	100	57.1	22.2	100
Lipolysis							
Tributyrin	91.8	5.9	100	100	100	100	100
No. of isolates	110	85	21	17	14	18	35

1. Yeast species. 1 = *Candida zeylanoides* 2 = *Debaryomyces hansenii* 3 = *Cryptococcus albidus*
4 = *Cryptococcus laurentii* 5 = *Rhodotorula glutinis* 6 = *Rhodotorula rubra*
7 = *Candida lipolytica*

2. Positive Response (%)

Figure 3.11 Effect of NaCl concentration on colony morphology after incubation at 25°C for 7 d

A 0% (w/v)	1 *Rhodotorula rubra*
B 5% (w/v)	2 *Sporidiobolus salmonicolor*
C 10% (w/v)	3 *Trichosporon pullulans*
D 15% (w/v)	4 *Candida lipolytica*


A
1
2
3
4
B
C
D

potential to cause contamination of the product.

2. The high fat content of the sausage might favour the growth of lipolytic yeasts (62.8). The extent of lipolysis, determined by measuring (mm) the zone of clearing on tributyrin agar, was found to vary within a species. A study of 20 isolates of Candida lipolytica (Table 3.6), for example, revealed three degrees of lipolysis (* - < 5 mm, ** - ≥ 5 mm, *** - > 6 mm). A correlation between degree of lipolysis and colony morphology was observed when the yeasts were grown on violet red bile glucose agar (VRBG) for 5-7 d at 25°C.
3. Assimilation of maltose (46.7) and glucose (100) could also be selective factors as both substrates are abundant due to breakdown of starch by enzymes of meat origin.
4. Assimilation of glycerol (72.7), a product of triglyceride metabolism, was also a common attribute of the yeasts isolated.
5. Assimilation of lactate (15.7), a product of post-mortem changes in the meat, was common with Candida lipolytica.
6. None of the yeasts assimilated starch but most were able to hydrolyse it to glycogen.
7. As would be expected, all the yeasts were able to grow at 5°C.
8. Formation of pseudohyphae (50.9) which was observed in Candida zeylanoides (100), Debaryomyces hansenii (30.6) and Candida lipolytica (100), might aid faster growth on the sausage surface.
9. Survival of the heat process either by formation of ascospores as in Deb. hansenii (81), or by cell lipid protection as in all Cryptococcus and Rhodotorula spp. (Figure 3.12) could be

Table 3.6 The lipolytic activity of isolates of Candida lipolytica

Isolate	Colony Morphology		Lipolysis[3]		
	VRBG[1]	OGY[2]	< 5 mm	⩾ 5 mm	> 6 mm
1		a			***
2		c		**	
3		b		**	
4		b			***
5	Colony is coiled	b			***
6		c		**	
7		c		**	
8		c		**	
9		c		**	
10		c	*		
11		c	*		
12		c	*		
13	Colony is smooth	c	*		
14	and glossy	d	*		
15		c	*		
16		a	*		
17	colony is smooth	b		**	
18	becoming rough,	c		**	
19	pitted or wrinkled	c		**	
20		c		**	

1. Violet Red Bile Glucose Agar

2. Oxytetracycline Glucose Yeast Extract Agar. Colonies described as: a = cream, shiny, lacy, flat; b = dull, furry, lumpy; c = smooth, shiny, small, d = smooth, dull, flat, spreading.

3. Lipolysis on Tributyrin agar determined by measuring zone of clearing.

Figure 3.12 Cell structure as observed with wet mounts (x400) prepared from growth on Oxytetracycline Glucose Yeast Extract Agar at 25°C for 7 d.

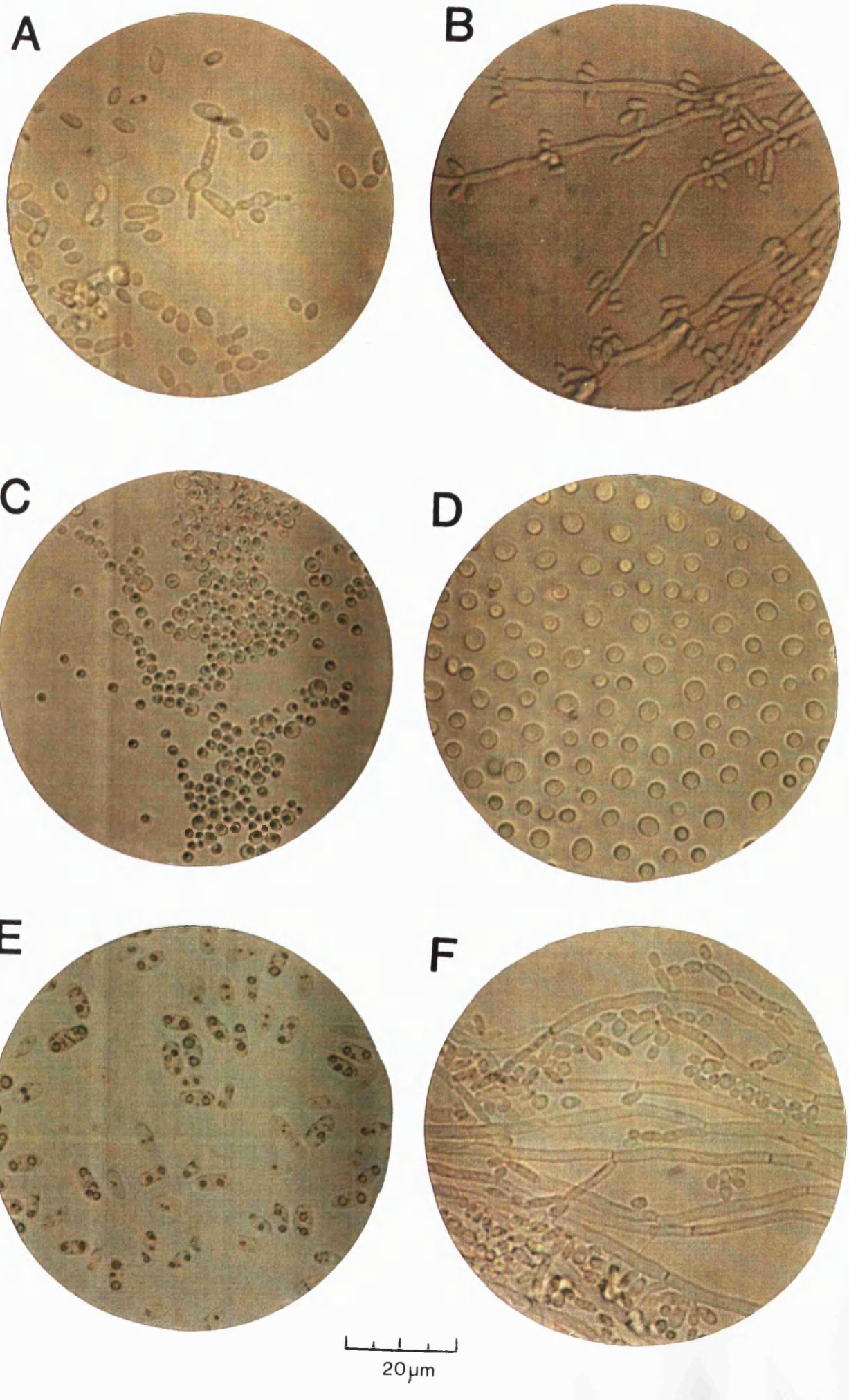
A, B *Candida zeylanoides*

C *Debaryomyces hansenii*

D *Cryptococcus albidus*

E *Rhodotorula glutinis*

F *Candida lipolytica*


A
B
C
D
E
F
20μm

another selective factor. Isolates of C. zeylanoides were occasionally observed to form thicker-walled, chlamydospore-type cells.

10. Cell shape may also influence colonisation. The ability to form spherical or small ovoidal/elongate cells as observed with Cryptococcus spp., Rhodotorula spp., Deb. hansenii and C. zeylanoides might increase the chance of survival in terms of reduced surface area when compared with the formation of mycelium as in C. lipolytica. The rate of subsequent colonisation of spherical cells might be enhanced in a nutrient rich environment because of the smaller volume to surface area ratio. Also single or small clusters of spherical cells will produce more colony forming units per se than longer, but fewer, strands of mycelium.

Further Observations of Yeasts

Certain imperfect yeasts with basidiomycetous affinity were investigated further by mixing with different isolates of the same species and growing for one to two months at either 4°C in the case of carotenoid yeasts or 15°C for isolates of Cryptococcus laurentii. It was hoped that these 'mating' experiments would induce formation of cells indicative of the teleomorph.

Carotenoid Isolates - The ten isolates studied were initially identified with Rhodotorula spp. but after growth on Gorodkowa agar strain 601 was found to be a ballistosporogenous yeast (Figure 3.13). There were five types of reaction; (A) copious formation of

Figure 3.13 Cells produced after mating carotenoid isolates. Wet mounts (x400) prepared from growth on Gorodkowa Agar after 6 weeks at 4°C

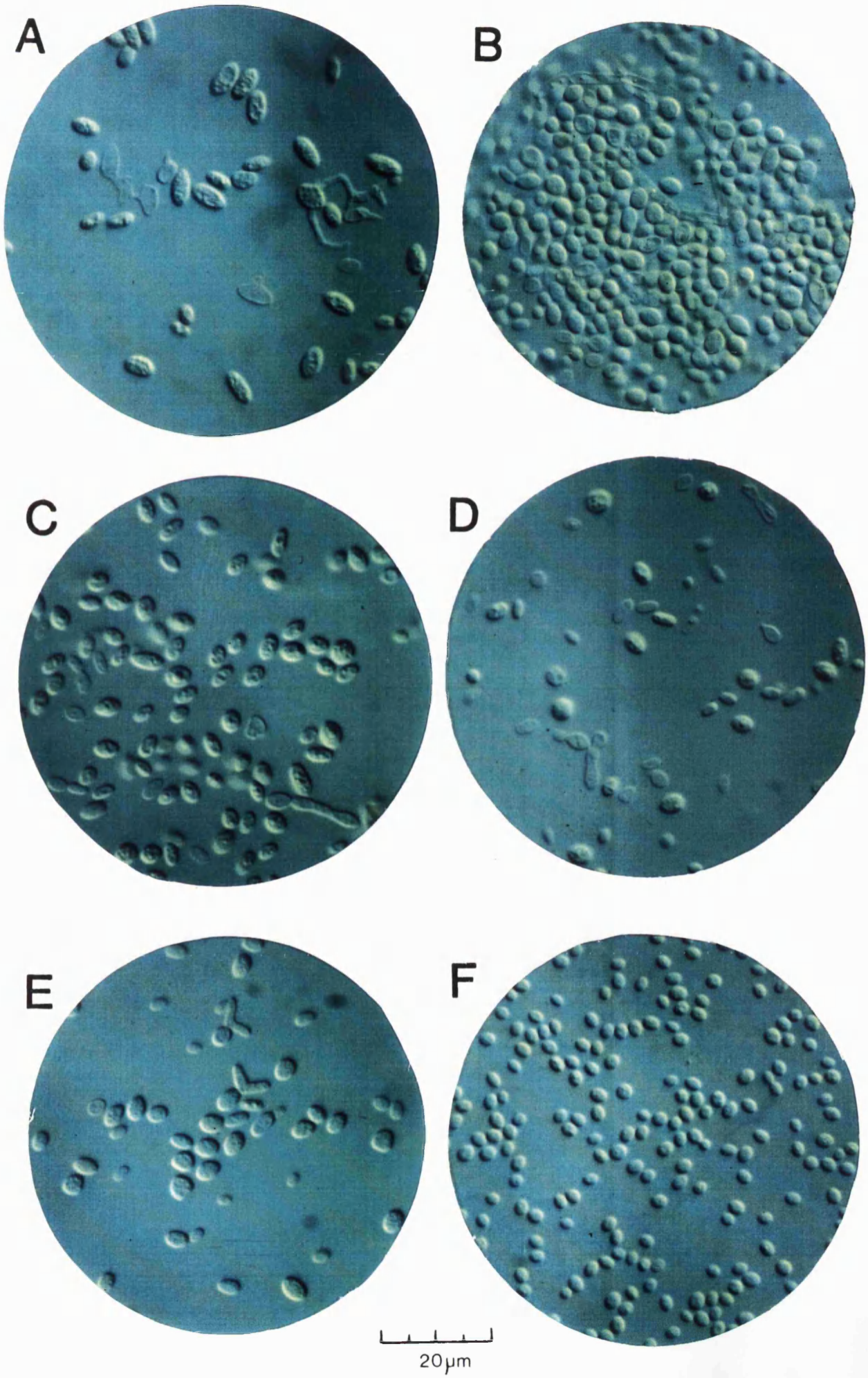
A Ballistospores, sterigmata

B Hyphae

C,D Germ tubes

E Triangular cells

F Single cells


A
B
C
D
E
F
20μm

ballistospores, metabasidia and basidiospores, (B) Formation of hyphae, (C) formation of germ tubes, (D) formation of triangular cells and (E) formation of single cells. The latter was the most common response and occurred with combinations of 600, 602, 603 and 604. Formation of triangular cells was a characteristic of 605. Germ tubes were observed with combinations of 606, 607 and 609. Hyphae formed when 608 was mixed with 605. Reaction (A) was induced when 601 was mixed with each of the isolates.

Cryptococcus laurentii - formation of hyphae was observed when either strain 1 or 5 were mixed with strains 2, 3, 4 or each other (Figure 3.14b). Chlamydospores formed when strains 1 and 5 were mixed. When the reactions above occurred the gross morphology of the colonies was smooth and glossy, becoming wrinkled, coiled and filamentous (Figure 3.14a). Mixing combinations of strains 2, 3, and 4 resulted in formation of single cells (Figure 3.14d) and the corresponding colony morphology was smooth, extremely glossy, entire and in the case of matings involving strain 3, slightly pink (Figure 3.14c). Such structures have been observed previously (Kurtzman, 1973; Laaser et al., 1988) and bear similarities to the basidiomycetous life-cycle yeasts, in particular to Tremella spp.

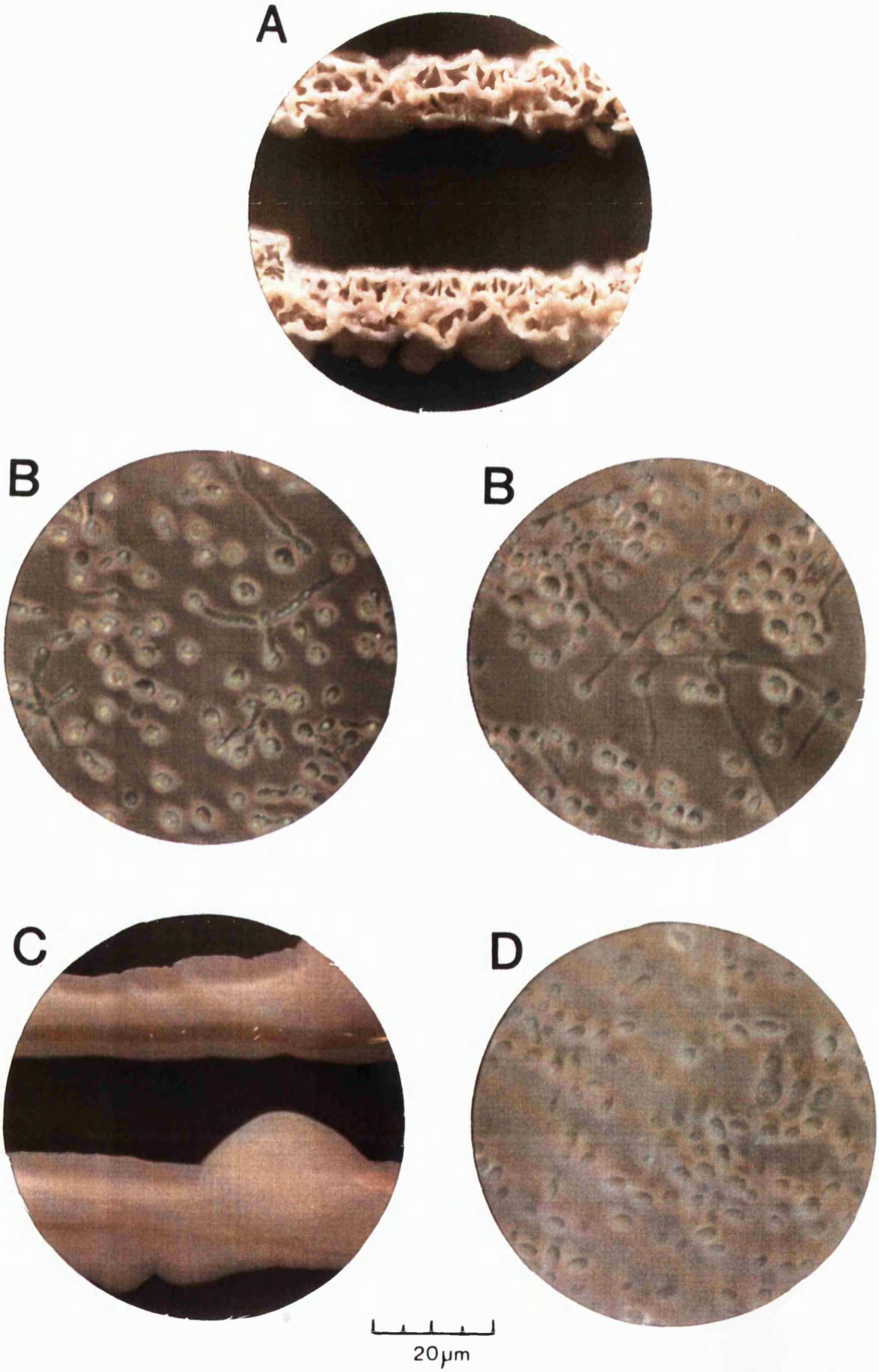
Figure 3.14 Colony morphology and cells produced after mating isolates of *Cryptococcus laurentii*

A Colony morphology when cells were as in B

B Conjugated cells, hyphae, chlamydospores

C Colony morphology when cells were as in D

D single cells


A
B
B
C
D
20µm

DISCUSSION

The survey of material from shops and the factory demonstrated that the flora of skinless sausage was similar both in range and types to that of the British fresh sausage. This is to be expected because both are based on a common meat emulsion. The major difference between the two types rests on one feature of skinless sausage production. Namely, the heat process which coagulates the peripheral proteins and eliminates most of the contaminants at the sausage surface. During storage (6°C) in the summer and to a lesser extent in the autumn and spring, surface yeast spoilage of sausages occurred as manifested by a general loss of colour, development of a stale odour and formation of cream colonies on the surface which coalesced into a generalised slime (Figure 3.3). It needs to be stressed that manufacturers are well aware of this form of spoilage during the warmer months but that, until now, contributory factors have not been investigated systematically.

Analysis of contamination during manufacture (i.e. raw ingredients through to packaged sausage) revealed that initially pseudomonads, Brochothrix thermosphacta and Enterobacteriaceae were the dominant organisms. In accord with the results of Dowdell and Board (1968), total bacterial populations varied between 10^5 and 10^7 cfu g^{-1} sausage meat. The presence of yeasts in the initial sausage mix was noted by Dowdell and Board (1971), Brown (1977), Banks (1983), and also Dalton (1984), who identified the yeasts which she isolated (Dalton et al., 1984). After the heat process numbers of bacteria were reduced by more than 96% and yeasts were

no longer detectable with the methods used in this study. Thus the increase in levels of organisms observed on the surface of the sausage immediately after packaging were a result of contamination, post-heat treatment. Counts in the core of the sausage were also reduced, but to a lesser extent so that proportions of particular organisms still reflected those in the original ingredients.

Possible sources of surface recontamination were the 'machine gun' used to remove the cellophane tubes from sausages and baffle plate (Figure 3.4), plastic crates in which sausages were stored prior to packing, the spray water used occasionally to moisten the surface so making the sausage easier to handle, the sorting bins and finally, the rubber gloves worn by the packers. Swabs and agar contact plates revealed that all of the surfaces were contaminated with the general flora of the original ingredient mix and that the rubber gloves were a particularly rich source of yeasts. Contact agar plates of the latter revealed a range of adventitious yeasts as well as those which caused spoilage. Even though the water-hose nozzle was heavily contaminated with bacteria, the water which it delivered was only very lightly contaminated.

Various attributes of the yeasts present at the time of spoilage were studied but no single elective factor was identified. In fact, the ability of these organisms to cause spoilage was probably due to a combination of physiological and morphological properties such that the yeasts could not only colonise the meat, but also remain viable in small numbers as would occur after the heat treatment and also generally on equipment. It was evident, however, that an improvement in general hygiene would curtail

initial sausage surface contamination, which originated from contact with the surfaces described above, such that spoilage would be delayed. The Hazard Analysis Critical Control Points (HACCP) technique which is now widely replacing traditional methods of Quality Control (QC) was chosen in a theoretical approach to this problem. By examination of raw materials, processes, practices, personnel, products, equipment and premises, HACCP highlights the actual or possible occurrence of potentially hazardous foods, pathogenic organisms and poor manufacturing practices resulting in dangerous, potentially dangerous or spoilt products (Bryan, 1981; Baird-Parker, 1987). A hazard analysis, therefore, estimates risks of producing, processing, or preparing foods by assessing all possible hazards and their likelihood of occurrence (Peterson and Gunnerson, 1974). If any hazards are identified, they are either eliminated or control measures are implemented to set acceptable limits for the hazard. Critical control points are established for phases of an operation where lack of control may cause a public health or spoilage hazard.

The public health record of skinless sausage is good, but as suggested by Legan (1981), post-processing contamination by a human carrier of Salmonella spp. for example, followed by storage at room temperature could be potentially hazardous because, owing to reduced microbial populations on the surface after heat treatment, the organism would face little competition. From the point of reduced shelf-life, control of post-processing yeast recontamination, particularly during the summer, might prevent effective competition of these organisms with lactic acid bacteria

and so curtail yeast spoilage.

Hazard analysis revealed several possible areas for control (Figure 3.4). The baffle plate of the 'machine gun' onto which each sausage was flung after tube removal and also the crates used for storage which were difficult to clean because of a layer of fat. Lengthy storage in the chill room (> 12 h), used to balance production and packaging, would lead to moisture loss from the sausage surface which through diminution of a_w, might subsequently support yeast and mould rather than bacterial growth. Although the water samples from the hose were very lightly contaminated, the nozzle was difficult to clean and was consequently a potential source of possibly hazardous contamination. The most important source of contamination, however, appeared to be the rubber gloves worn by the packers. Presumably, more stringent cleaning procedures, more frequent changing of gloves, and the adoption of crates made from materials easier to clean, would reduce the amount of contamination of the sausage. Conversely, should such procedures merely reduce bacterial contamination and therefore increase the yeast to bacterial ratio, then yeasts might compete effectively and cause spoilage throughout the year and not just in the warmer months. For this reason, any change in cleaning regimes should be rigorously monitored for effectiveness against both bacteria and yeast. Occurrence of yeasts could be determined using oxytetracycline supplemented Violet Red Bile Glucose Agar (VRBGO). As described in Chapter 2, this medium supports the growth of yeasts from meats which can be identified quickly by observing colony appearance after 5 d at 25°C.

By studying the development of the spoilage flora, it was found that the pasteurisation effect persisted througout storage at 6°C in both the core and coat of sausage and that growth of yeasts was considerable whereas that of bacteria was negligible (Figure 3.6). As with British fresh sausage, a Gram-positive flora and yeasts were always selected during storage. In summer and autumn, yeasts on the surface of skinless sausage stored at 6°C greatly outnumbered bacteria and accounted for approximately 90% of the flora - they were responsible for spoilage (yellow slime). Indeed, as suggested in Chapter 1, it appeared that the ratio of yeasts to lactic acid bacteria was of importance in determining the type of spoilage. In winter the bacteria which outnumbered yeasts were the cause of spoilage (souring) whereas in spring when numbers of each were similar, yeasts only occasionally caused spoilage. It was concluded that when the ratio of lactic acid bacteria to yeast was $\leqslant$ 3.2 : 1 (and in most cases it was less than 1 : 1), a feature of summer and autumn samplings, then spoilage of the commodity was due to yeasts.

Reasons for the increased yeast growth during the summer months and ability to compete effectively with lactic acid bacteria so resulting in spoilage have not been identified. Yeast spoilage occurred even though the surface was dominated by bacteria immediately after packaging. At this stage, yeasts detected on the surface comprised 'casual' contaminants as well as those types which eventually colonised the surface and caused spoilage. It would seem that during warmer weather when general contamination levels of both ingredients and equipment were higher than in the

winter, yeasts were able to compete effectively with lactic acid bacteria. At this time, when higher temperatures cause rapid drying out of meat debris on equipment, yeasts which can survive at low a_w's may outgrow bacteria resulting in a higher level of post-processing contamination with yeasts. As noted in Chapter 1, yeasts were the dominant flora on surfaces with low a_w in an abattoir chilling room (Thompson *et al.*, 1985).

The following spoilage yeasts were ubiquitous on processing equipment; *Candida zeylanoides*, *Debaryomyces hansenii*, *Cryptococcus* spp., *Rhodotorula* spp. and *Candida lipolytica*. The order of colonisation observed during storage (Figure 3.8) was accentuated during the summer months when yeast growth was most pronounced. In terms of metabolic attributes, all of these yeasts appeared well-suited to growth in skinless sausage (Table 3.5). The sausage provides a large reservoir of carbohydrate and, although no yeasts were able to assimilate starch, many could assimilate glucose and maltose which result from amylase activity of meat and rusk origin (Abbiss, 1978). Many yeasts exhibited extracellular amylase activity and were able to produce glycogen from starch. Abbiss (1978) noted only sporadic occurrence of glucoamylase activity in sausage and was of the opinion that it was produced by microorganisms as the activity was not detectable in either meat or rusks. He suggested that glucoamylase was produced solely in response to a limited supply of nutrients and that, under such conditions, yeast-mediated amylase activity might release additional carbon sources. In this study all of the main yeasts were able to hydrolyse starch. Lactate, the principal product of

post-mortem glycolysis of meat (Lawrie, 1976), was assimilated by C. lipolytica (91.4%) and Deb. hansenii (17.7%), but not by the other yeasts. Gluconate, which occurs in meats as a consequence of microbial metabolism (Nychas, 1984; Nychas et al., 1988) was only assimilated by Cryptococcus and Rhodotorula spp., probably another example of selective nutrient capture. Fats constitute 10 - 40% of the sausage (Hankin, 1986) and represent a large reservoir of carbon and energy in the form of triglycerides. Yeast-mediated lipolytic activities (62.8% of the isolates were positive), may release fatty acids and glycerol by triglyceride metabolism. It was noteworthy that 72.7% of the yeasts assimilated the latter. Brown (1977) attributed fatty acid accumulation to microbial degradation of fat, a role later associated with yeast populations by analysis of volatile fatty acid content of sausage (Leads, 1979). It was apparent from these observations that the yeasts which colonised the sausage during storage were well adapted to growth in this environment. Growth rates (expressed as generation times) of the dominant yeasts at spoilage - C. zeylanoides, Deb. hansenii and C. lipolytica - in a laboratory meat broth were ca. 1.5 - 2 h, and faster than either Cryptococcus or Rhodotorula spp., which grew at ca. 3 h. Growth in sulphited (500 μg ml^{-1}) broth at pH 6 did not effect growth rates to any great extent but an extended lag phase was observed with Cryptococcus albidus and Rhodotorula glutinis. Perhaps the slower growth rate together with a protracted lag phase resulted in the demise of the latter two genera during the succession noted in Figure 3.8. Although all of the yeasts were able to tolerate concentrations of sulphite present in sausage,

growth of only three of the yeasts was correlated with an increase in bound sulphite, namely C. zeylanoides, Deb. hansenii and Rh. glutinis. The first two of these yeasts produced acetaldehyde, a known sulphite binding compound (Burroughs and Sparks, 1964, 1973; King et al., 1981). The amount produced, however, accounted for only a small percentage of the bound sulphite (17 - 26%). This latter observation contrasts with that of Dalton (1984), who found that acetaldehyde was the major binding compound in sausage accounting for ca. 40% after storage at 4°C and 70% at 15°C. She concluded that acetaldehyde was an important but not the sole sulphite binding compound in sausage. Brown (1977) suggested that rusk and starch provided a reservoir of sulphite binding compounds. Results from the present study appear to confirm that a variety of compounds were responsible for the increase in bound sulphite and that yeasts were only partly responsible. Indeed, it is doubtful that the production of such compounds would confer any advantage as far as colonisation is concerned, particularly as C. lipolytica neither bound sulphite nor produced acetaldehyde but was still dominant at spoilage. In conclusion, the slower growth rates and extended lag phases of Cryptococcus albidus and Rhodotorula glutinis may have resulted in their failure to contribute successfully to the colonisation of the sausage surface during storage. A combination of growth rate, substrate utilisation and cell morphology were probably responsible for the dominance of Candida zeylanoides, Deb. hansenii and C. lipolytica at spoilage. It is noteworthy that these three yeasts were dominant in the original ingredient mix and were also the most frequently isolated

yeasts on other makes of skinless sausage and in British fresh sausage.

The ability to reproduce sexually and asexually and to form structures other than single cells (Figures 3.13, 3.14), may enable the yeast to adapt more quickly to a change in environment. Few of the yeasts isolated from ingredients and sausage were teleomorphs, indeed many were assigned to the genera Candida, Cryptococcus and Rhodotorula. The two latter anamorphic genera have basidiomycetous teleomorphs (Kreger-van Rij, 1984). Indeed, perfect yeasts such has Sporidiobolus salmonicolor and Sporid. ruinenii were isolated from the raw ingredients but not from the finished product. Studies on carotenoid yeasts and also on Cryptococcus laurentii revealed that, by mixing isolates, different cell forms could be induced (Figures 3.13, 3.14). These structures often bore similarities to fractions of basidiomycetous life-cycles. For example, cells formed conjugation tubes and hyphae in the case of Cryptococcus laurentii (Figure 3.14). The anamorphic genus Cryptococcus typically contains inositol-positive, non-filamentous yeasts with regular, spherical/ovoid cells; however, certain species have been allocated basidiomycetous teleomorphs in the genera Filobasidiella, Filobasidium and also Rhodosporidium. Kurtzman (1973) mixed strains of Cryptococcus laurentii and observed cells that formed conjugation tubes which gave rise to hyphae with chlamydospores. He noted that such structures were characteristic of the genus Tremella. In fact, the inositol-positive Tremella spp. are all included in the Cryptococcus key (Kreger-van Rij, 1984). Recent work by Laaser et al. (1988) used DNA homology analysis to

investigate yeasts which had exhibited, albeit infrequently, a mycelial phase typical of Tremella spp. Although the yeast would be classically identified with a species of Cryptococcus (Kreger-van Rij, 1984), they observed hyphae with dolipores and cup-shaped parenthesomes. These structures are highly indicative of the teleomorph genus Tremella. Despite the failure to observe any sexual structures, these authors were convinced that the yeast showed a stronger tremelloid than cryptococcalean relationship. Indeed, when the isolates of Cryptococcus laurentii from the present study were mixed and cells formed conjugation tubes and hyphae, the corresponding colony morphology was that of the tremelloid yeast culture described by Laaser et al. (1988). Among taxonomists, there is much controversy concerning delimitation of the anamorph genus Cryptococcus. In the present study yeasts were identified with the anamorph unless sexual structures were observed. Many yeasts rarely form such structures, however, and isolation and subsequent study present extremely artificial growth conditions when compared with natural habitats. Thus, any yeast assigned to an anamorphic genus was carefully observed for the appearance of any unusual structure typical of a teleomorphic state.

CHAPTER 4

LINEAR DISTRIBUTION OF YEASTS ON POULTRY PROCESSING EQUIPMENT

The composition and range of the contaminating yeast flora on equipment and products in two poultry processing plants were investigated. The overall objectives were (1) to establish whether or not there was a linear distribution of yeast types on equipment, and (2) to examine the relationship between flora on equipment and, after further processing and storage, on product contamination.

INTRODUCTION

Microbial spoilage of poultry was of little commercial concern when the majority of broiler chickens were deep frozen from the time of production until preparation for consumption. During the last decade, however, changes in consumer attitudes towards food and in particular to meat (Breidenstein, 1988) have resulted in an increased demand for fresh (chill) broilers. For example in the UK, whole chicken consumption per annum has remained at about 250 000 tons for the last 10 years, but the proportion of fresh whole chicken consumed has progressively increased to about 120 000 tons - 50% higher than it was in 1981 (Hood et al., 1988). The advantages of fresh over frozen broilers are that taste and natural bloom - a subjective assessment of skin colour and texture - are retained. The frozen broiler is at best very pale due to ice crystal formation within the fibres and at worst yellow, due to slow freezing and formation of larger crystals outside the fibres. Chicken can be kept in good condition for months if freezing is prompt and rapid and the storage temperature sufficiently low (Frazier, 1967). Storage of fresh poultry, however, is usually for a few days only. The closer the temperature is to zero, the longer the birds can be stored without spoilage occurring (Barnes and Thornley, 1966). Before storage, eviscerated fresh chicken are chilled rapidly, either by immersion in iced water, which leaves the bird wet, or by cold air, which leaves the bird relatively dry and is the preferred method. There have been sporadic reports of

spoilage of air-chilled chicken; an alcoholic aroma develops and the skin becomes pink as a result of microbial growth. These observations are indicative of yeast growth and indeed, the drier surface of the carcass - hence the lower a_w - would be expected to support yeast rather than bacterial growth (see Chapter 1).

The bacterial associations of fresh chicken have been studied extensively, especially the pseudomonads responsible for spoilage (Barnes and Thornley, 1966; McMeekin, 1975, 1977). Likewise, attention has been given to food-poisoning organisms such as Salmonella, Clostridium and Campylobacter spp. (Cunningham, 1982; Genigeorgis, 1987). Indeed, Hood et al. (1988) suggested that the rising incidence of Campylobacter enteritis during 1981-1986 could well be a reflection of the increasing proportion of fresh chickens consumed over that period (32% higher in 1986 than in 1981). In this instance the organisms remained viable at chill but died out with deep frozen storage. The increasing number of human salmonellosis outbreaks associated with poultry has stimulated a greater public awareness of the need for improved standards of hygiene when preparing this meat. In particular, the informed cook is now familiar with the dangers of inadequate cooking and contact of other foods and equipment with raw poultry (Horrox, 1988).

The microbial condition of the processed carcass is related to the numbers and types of microorganisms transferred to and from processing equipment by the bird and to the effectiveness of control measures within the plant (Mead, 1982). Attempts to diminish microbial contamination of the ready-for-sale carcass have been directed at two targets. Firstly, studies of microbial

contamination of equipment and carcasses during processing have identified particular processing stages where critical control is essential (Thomas et al., 1987) and, in many instances, adaptions to equipment design have had to be made to avoid build-up and transfer of contamination (Anon.,1988b; Purdy et al., 1988). Secondly, post-processing irradiation of carcasses has been shown to reduce levels of bacterial contamination (Anon., 1987; Hanis et al., 1989).

All of these methods reduce the numbers of bacteria but as yet, the effect on yeast numbers has not been investigated. It was concluded in Chapter 1 that conditions which result in a decrease in bacteria may well lead to a yeast to bacteria ratio of 1 : 1, a situation where yeasts may compete more effectively with bacteria such that they cause spoilage. An example of the importance of the yeast to bacteria ratio was discussed in Chapter 3. The heat process in skinless sausage production caused a dramatic reduction in levels of contamination. Yeasts became the dominant organisms and frequently caused spoilage during the warmer months when they competed successfully with lactic-acid bacteria.

As noted above, there have been anecdotal reports of yeast spoilage of air-chilled chickens, presumably because the lower a_w of the broiler surface and of meat debris on equipment in air-chillers, will support yeast rather than bacterial growth. Recent work by Hughes and Patterson (1988) suggested that irradiated chicken may well exhibit yeast spoilage since, after a dose of 2.5 kGy, these organisms were the major component of the flora of chicken skin. Johannsen et al. (1984) found no reduction

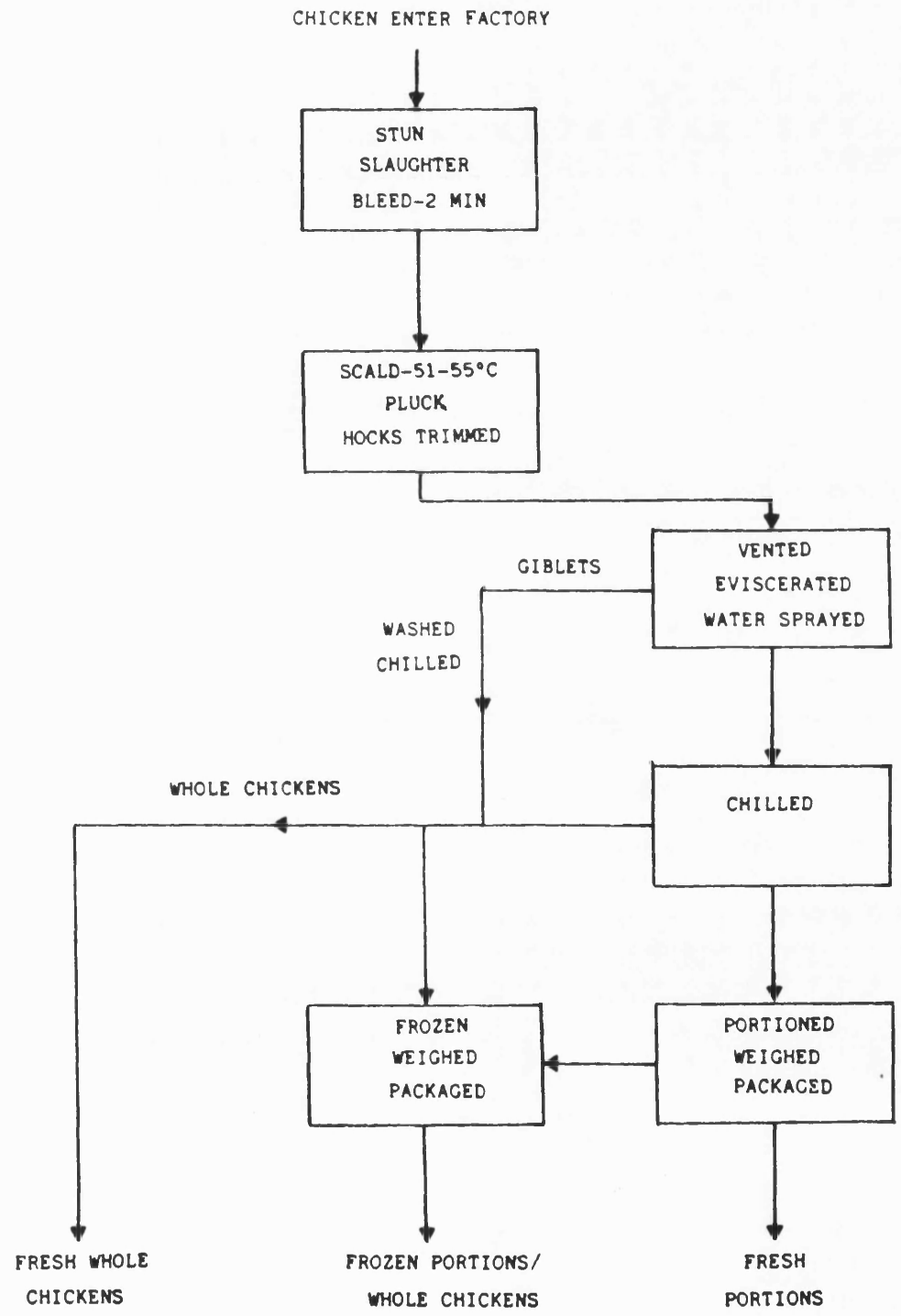
in yeast cell numbers when minced meat was radurised with a dose of 2.5 kGy and a marked increase after 14 d storage at 4°C.

It is probable that irradiation will soon be adopted to reduce levels of salmonellae contamination of fresh broilers so that the incidence of food-poisoning outbreaks is diminished. In view of the above observations, it was concluded that a study of yeast contamination on equipment and carcass throughout processing and storage was required.

Poultry Processing

Birds are normally starved before slaughter so that faecal contamination during transport and processing is minimised. The birds are normally transported in a multi-cage system (10-15 per cage depending on bird size) where the base of the cages are perforated. This prevents the birds from sitting in an accumulation of their own droppings, but leads to contamination of birds at lower levels in the cage system (Bryan et al., 1968). To prevent cross-contamination of flocks, the cages must be thoroughly cleaned and disinfected before being used for another journey. Further contamination occurs, due to flapping of wings and a general struggle, as birds are transferred to the processing line (Patterson, 1973). Throughout processing, birds are shackled (hung by their legs). The spread of airborne contaminants is minimized by completely separating the unloading bay from subsequent processing areas. There are six main stages in processing (Figure 4.1); these are normally done in different rooms to curtail the spread of microorganisms within processing plants.

Figure 4.1 Flow diagram of a poultry processing plant



1. After shackling, the birds are electrically stunned and slaughtered by severing the blood vessels in the neck. Bleeding for at least 2 min occurs, during which time carcasses are sprayed with water to reduce the amount of blood in the slaughter area.
2. The freshly slaughtered birds are scalded (2 min at 51°C) in order to loosen the feathers. This is termed 'soft-scalding' and is the preferred method for fresh poultry since cuticle damage is minimal and the ultimate appearance and 'quality' of the bird is preserved. Immediately after scalding the birds pass through a series (usually 2) of mechanical plucking machines. Feathers are removed by the flailing action of rubber fingers. With the passage of warm, wet carcasses through the plucking machines, conditions soon become suitable for microbial growth particularly as the flap doors situated at the ends of the machines and the rubber flails are difficult to clean and disinfect effectively. The plucking machines and surrounding machinery are sprayed with chlorinated (20 ppm) water. Purdy et al. (1988) showed that Staphylococcus aureus became endemic on the rubber flails after changes in machinery design and a reduction in the level of chlorination. The increase in contamination resulted in higher bacterial counts on the final carcasses. Trimming of hocks is completed before a carcass enters the next room.
3. Removal of viscera involves several stages and inevitably leads to further contamination of carcasses with intestinal organisms. Automated evisceration eliminates human error and

handling whilst providing facilities for washing and disinfecting the implements. Unfortunately, the equipment requires birds of a uniform size - an objective that is not always realised - in order to avoid rupture of the gut. The head and neck are removed and both the outside and the cavity of the carcass are thoroughly spray-washed. Sometimes, rotating rubber flails are used to assist cleaning of the skin, but the flails rapidly become contaminated and may lead to cross-contamination. According to Mead (1982), spray-washing reduces the carcass contamination by less than 10-fold because many of the remaining organisms are trapped in feather follicles and other inaccessible parts of the bird. It is important, however, to clean the carcass in order to facilitate the hygienic operation of the water-immersion chilling systems; by minimizing the number of organisms being introduced to this stage the opportunities for subsequent cross-contamination between carcasses is curtailed (Van Schothorst et al., 1973). After evisceration, edible organs are dealt with separately to avoid dissemination of the gut flora.

4. As the inner muscle temperature is invariably above 30°C after evisceration, it is necessary to chill the carcass immediately in order to limit the growth of both spoilage and pathogenic organisms. There are two methods used for this. One involves immersion of the carcasses in iced water and the other, chilling of shackled carcasses in cold air. The water-immersion chillers investigated in this study were of the archimedes, counter-flow design. Unshackled, washed carcasses are dropped

into one end of the chiller which contains a continuously rotating screw, and ice-chilled (6°C), chlorinated (20 ppm) water is added to the other end. In this way carcasses are agitated and moved against the flow of water by the screw so that, at the end of chilling (30 min), the carcasses are in contact with the fresh water. During this process organisms are removed from the inner and outer surfaces of the carcass by the water and also by attrition. The addition of chlorine to the water reduces the number of microorganisms and therefore the amount of cross-contamination (Thomson et al., 1979). Continuous overflow of water from the chiller also contributes to the control of contamination. Large processing plants use water-chilling for carcasses to be frozen and air-chilling for those intended for fresh sale. The latter involves shackled, washed, eviscerated carcasses being hung in a chill-room (0 ± 2°C). It has been reported that the dry skin of an air-chilled carcass is important in delaying the growth of spoilage organisms due to its diminished a_w. Although this method avoids the cross-contamination seen with water-chilled carcasses, Mead (1975) demonstrated that with the lower scalding temperatures used to prevent damage to the cuticle, and the absence of the washing effect noted above, air-chilled birds were more highly contaminated than their water-chilled counterparts. He also showed that air- and water-chilled carcasses wrapped in oxygen-permeable film and held at 2°C had a similar shelf-life.

5. After chilling, carcasses enter another room (Figure 4.1) where they are trussed and graded before packs of giblets are added

and the birds packaged. Portioning of chickens is also done in this room which is maintained at a chill temperature. A network of conveyors is used such that wings are removed then breasts from which filleted breasts may be prepared, and finally, the legs are removed. The design of the cutting implements means that stringent cleaning procedures need to be done at frequent intervals to avoid build-up of contamination and transfer to portions. It is notable that the cut-muscle edge and feather follicles always support the highest numbers of spoilage microorganisms during storage (Mead, 1982).

6. Whole and portioned chickens intended for sale as frozen products are blast frozen and stored at -20°C before dispatch. Those intended for the fresh (chill) market are stored in a chiller at 4°C.

Treatments to Eliminate Pathogens and Extend Shelf-Life

As suggested by Thomas et al. (1987), pathogenic organisms should ideally be eliminated from the bird before slaughter to prevent contamination of the plant. The work of Notermans et al. (1975) showed that contamination occurring early in processing was not readily removed, resulting in an increased load on the final carcasses because the chlorine wash was less effective. Purdy et al. (1988) investigated the effects of a change in design of the plucker machine and in chlorine concentration of spray-water on contamination levels. They concluded that only small changes in processing methods were required to achieve marked improvements in the microbial contamination flora of poultry processing plants.

Various post-processing treatments for extending the shelf-life of fresh poultry and eliminating food-poisoning bacteria have been sought (Thomson et al., 1976; Islam et al., 1978; Anon., 1988b). Thomson et al. (1976) eliminated salmonellae from chicken carcasses by using a hot dip in 1% succinic acid + 50 mg l^{-1} of chlorine. The treated carcasses were chilled in chlorinated water. Although effective in microbiological terms, the skin was discoloured such that the carcass had a slightly cooked appearance. Recently, a patented system (Anon., 1988b) which involves immersion of carcasses in acidified, scalding, chilling and/or spray water containing a surfactant has been developed. It was claimed that this invention was a "commercially viable" means of controlling cross-contamination from one carcass to another and also for control of salmonellae organisms on the carcass. Another possible method of preservation is that of 'super-chilling' where the carcasses are stored at -2°C (see Chapter 1). Although this process is unlikely to be widely employed due to the enormous power-cost involved, observations suggest that yeasts could cause problems. Storage at this temperature where, any free water present may become frozen whilst the meat does not, will influence the a_w of the prduct. Barnes et al. (1978) found that yeasts which are normally less affected by low a_w than bacteria formed a much higher proportion than usual of the total flora of polyethylene-wrapped, air-chilled turkeys.

The use of irradiation to eliminate pathogenic organisms and extend shelf-life of raw meat products has been studied widely since the middle 1950's. Ingram and Thornley (1959) found that the

flora of chicken exposed to > 2.5 kGy was dominated transiently by yeasts, the final spoilage flora was mainly bacteria. When chicken were pretreated with chlorotetracycline, yeasts made a larger contribution to the spoilage flora. This would be expected since yeasts are known to be more resistant to tetracyclines than bacteria (Njoku-Obi et al., 1957). High doses of irradiation have detrimental effects on both taste and appearance of chicken and these are accentuated during storage (Coleby, 1959). This author found that chicken exposed to low doses had an extended shelf-life when stored at chill temperatures. It is evident from the review by Mossel (1987) that dose requirements for the elimination of bacterial enteric pathogens from fresh meats and poultry by irradiation - without adverse effects on meat quality - have been determined as accurately as is achievable (Dyer et al., 1966; Davies, 1976; Prachasittisadki et al., 1984; Anon., 1987; Hanis et al., 1989). The two major problems preventing the application of this method to fresh meats are; first, the anxious consumers' worries regarding the safety of radurised food, especially in the wake of recent problems in America and Russia, and second, from a commercial point of view, concerns that importing countries will deny entry of products treated by radurisation because of 'radiophobia'. In conclusion, it would appear that future emphasis on decontamination of raw meats will centre around the use of a 'pasteurising' dose of irradiation. It is obvious from current literature and frequency of food-poisoning outbreaks (Bryan, 1981; Hood et al., 1988) that current methods of production and distribution of fresh meat and poultry are inadequate to protect the consumer.

As discussed in Chapter 1, conditions that are acidic or of low a_w commonly select for yeast growth. It was also suggested that any treatment which causes a reduction in bacterial numbers enables yeasts to compete and cause spoilage. Problems associated with production and distribution of poultry are increasingly highlighting the requirement for a safer product. This has necessitated investigation of possible changes in processing or alternatively, post-processing treatments. It is clear that scant attention has been paid to how such treatments may affect yeast populations and yet, yeasts have been implicated as spoilage organisms after 'super-chilling' and irradiation of poultry (Barnes et al., 1978; Ingram and Thornley, 1959; Hughes and Patterson, 1988).

The present study aimed to investigate the contribution of yeasts to the flora on carcasses and equipment of two processing lines. The effect on yeasts of post-processing treatments was studied by examining the flora of packaged whole and portioned, fresh and frozen chickens after chill and frozen storage and also after irradiation.

MATERIALS AND METHODS

FACTORY SURVEY

To determine types and levels of contamination on poultry processing lines (Figures 4.1, 4.4), equipment was sampled using calcium alginate swabs (Oxoid). Water samples (10 ml) were removed, where appropriate, with sterile plastic Pasteur pipettes (Sterilin) and transferred into sterile plastic universals (Sterilin). Petri dishes containing Oxytetracycline Glucose Yeast Extract Agar (OGY, Oxoid) or Plate Count Agar (PCA, Lab M) were used as 'contact plates' for investigation of the contamination on the hands of workers involved in either evisceration or packing. Air samples (Biotest RSC Centrifugal Air Sampler) from each of the processing chambers were obtained (40 l min^{-1}) using strips containing OGY. Chicken carcasses or portions were placed aseptically into sterile bags and, together with swabs and water samples, transported to the laboratory in an insulated box containing ice packs. Air-sample strips and 'contact plates' were incubated at 25°C for 5-7 d and chicken meat was stored immediately at 4°C until sampling. Swabs were dissolved in 10 ml quarter-strength 'calgon' Ringer's solution (Oxoid) to give a 10^{-1} dilution from which further serial dilutions were made. Samples (0.1 ml) of appropriate dilutions were spread-inoculated (6 replicates) over the surface of Petri dishes containing (a) OGY or (b) Violet Red Bile Glucose Agar (Lab M) suppemented with 100 mg l^{-1} oxytetracycline (VRBGO). As described in Chapter 2, VRBGO was adopted as a differential medium for

studies of meat yeasts. Incubation was at 25°C for 5-7 d.

SAMPLING AND STORAGE OF CHICKEN

Fresh chicken was either sampled immediately or after storage at 6°C for 1 week; frozen chicken was sampled after overnight storage (1-5°C) in a refrigerator as recommended by Barnes et al. (1973). For long term storage experiments, portions (3 replicates) were stored at -18°C. In all cases storage temperatures were monitored constantly (PTI 05 - FSA Laboratory Supplies, Loughborough).

Chicken portions (3 replicates) were sampled by removing aseptically skin (10 g) and meat (10 g) from a cut edge or from the centre of a portion. These were each placed in sterile stomacher bags with 90 ml sterile quarter-strength Ringer's solution (Oxoid) and homogenised for 60 sec in a Colworth Stomacher 400 (Seward, London). The homogenate was transferred to a sterile medical flat (200 ml) and allowed to settle into three layers. A range of serial dilutions (10 ml) of the middle layer were prepared in sterile diluent. The dilutions were shaken vigorously to ensure homogeneity and plated (4 replicates) onto OGY and VRBGO for yeast and, Plate Count Agar (Lab M) for general bacterial counts. Incubation was at 25°C for 5-7 d.

The visceral cavity of chickens was sampled by swilling with sterile diluent (1 l) and making further dilutions from this (Barnes et al., 1973; Notermans et al.,1975). Samples (10 g) of skin from the breast region and from under the wings and also meat from the breast and leg region were removed using a sterile scalpel

and homogenised as described above. Giblets (10 g), which were packaged separately in the factory, were homogenised with 90 ml sterile quarter-strength Ringer's solution and dilutions prepared as above.

Frozen Storage

Three chicken portions were removed prior to freezing to obtain initial yeast and bacterial counts. Neighbouring portions (12) on a conveyor belt were marked with a spot of cochineal food colouring and removed after transit through a blast freezer (-18°C). They were placed in an insulated box containing ice packs (stored at -18°C overnight) and transported to the laboratory. Three were thawed overnight (5 ± 1°C) the skin and the cut muscle edge were sampled as above. The remaining portions were stored at -18 ± 2°C and three were removed and sampled at monthly intervals. Incubation of Petri dishes (4 replicates) containing either PCA or OGY was at 5, 15 and 25°C.

Irradiation of Chicken Portions

Fresh (18) and frozen (18) portions were placed aseptically in stomacher bags which were taped and labelled. 'Continuous' type γ-irradiation (Isotron, Swindon, England) was used at a dose rate of 10.0 kGy h^{-1}. Fresh and frozen portions (3 of each) were given the following doses; 1.25, 2.50, 3.75 and 5.00 kGy. The remaining control portions (12) were untreated. After irradiation, the bags were placed in an insulated box containing ice packs and transported to the laboratory.

In order to obtain initial counts of yeast and bacteria present on the skin before irradiation or storage, untreated fresh portions (3) were sampled immediately and untreated frozen portions (3) were allowed to thaw overnight (5 ± 1°C) and then sampled. Skin (10 g) was removed aseptically and homogenised with 90 ml sterile quarter-strength Ringer's solution. Appropriate serial dilutions (0.1 ml) were spread-inoculated (4 replicates) on the surface of Petri dishes containing either Plate Count Agar (PCA, Lab M) or Oxytetracycline Glucose Yeast Extract Agar (OGY, Oxoid) for a general bacterial or yeast count respectively. Incubation was at 25°C for 5-7 d.

The remaining frozen portions were stored at -18 ± 2°C for one week and the fresh portions at 5 ± 1°C for 3 d before being sampled as described above. In all cases storage temperatures were monitored constantly (PTI 05 - FSA Laboratory Supplies, Loughborough).

RESULTS

TYPES OF YEAST

In the present study a total of 210 yeasts were isolated from equipment in the two poultry processing plants and from poultry meat. Violet Red Bile Glucose Agar supplemented with oxytetracycline (VRBGO) was used as a screening, differential medium and the simplified classical key (see Chapter 2) enabled rapid identification. The majority of species belonged to the six 'meat genera' mentioned in Chapter 1. Namely: Candida, Cryptococcus, Trichosporon, Rhodotorula, Debaryomyces and Pichia (Table 4.1). The proportions of particular genera and species differed from those of skinless sausage (see Chapter 3). As discussed previously, there was a linear distribution of yeasts on equipment in poultry processing plants (Table 4.2). Filamentous yeasts, notably Trichosporon cutaneum, were most frequently isolated in the early stages of processing whereas smooth types became dominant post-evisceration (Figure 4.4). Whole and portioned chicken were contaminated with a range of yeasts that were also found to contaminate equipment in the final processing rooms. The most commonly isolated were: Candida japonica, Tr. cutaneum, Cr. laurentii, Candida tropicalis, C. lipolytica, Rhodotorula glutinis and Sporobolomyces salmonicolor. Colony morphology of these yeasts on Oxytetracycline Glucose Yeast Extract Agar after 5 d at 25°C is illustrated in Figure 4.2 and wet mounts (x400) revealed cell morphology (Figure 4.3). It is important to note that whilst the

Table 4.1 Yeasts isolated from poultry and processing equipment

Genus Species	1*	2	3	4	5	6	7	8
Bullera								
alba					+	+	+	
Candida								
diddensiae							+	
inconspicua								+
japonica	+	+	+	+	+		+	+
lipolytica	+	+	+	+	+	+	+	+
mesenterica								+
parapsilosis	+					+	+	+
sake		+						
scottii						+		+
solani								+
steatolytica	+							
tenuis				+	+			
tropicalis	+	+	+					
vini							+	+
zeylanoides					+	+		+
Cryptococcus								
albidus	+	+	+	+	+	+	+	
laurentii	+	+	+				+	+
Debaryomyces								
hansenii		+		+	+	+	+	+
Geotrichum								
candidum				+		+	+	+
Pichia								
burtonii				+	+			
Rhodosporidium								
infirmominiatum						+	+	+
Rhodotorula								
glutinis	+	+	+	+		+	+	+
minuta							+	+
rubra				+		+	+	
Sporidiobolus								
pararoseus		+					+	
ruinenii		+					+	+

Table 4.1 continued

Genus Species	1*	2	3	4	5	6	7	8
Sporobolomyces								
holsaticus	+					+	+	
salmonicolor	+	+	+					+
Tremella								
globospora	+						+	+
Trichosporon								
aquatile							+	
capitatum						+	+	+
cutaneum	+	+	+	+	+	+	+	+
fennicum						+		
figueirae						+	+	

* 1 = whole chicken (fresh)

2 = portions (fresh)

3 = portions (frozen)

4,5,6 = processing plant A

7, 8 = processing plant B

Figure 4.2 Colony morphology of yeast isolates on Oxytetracycline Glucose Yeast Extract Agar after 5 d at 25°C.

A *Candida japonica*

B *Trichosporon cutaneum*

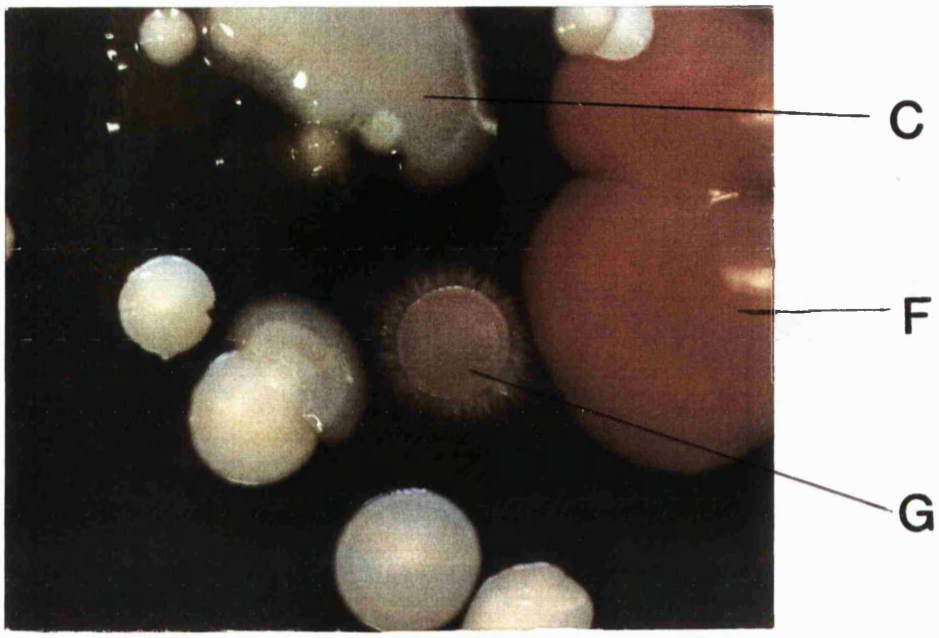
C *Cryptococcus laurentii*

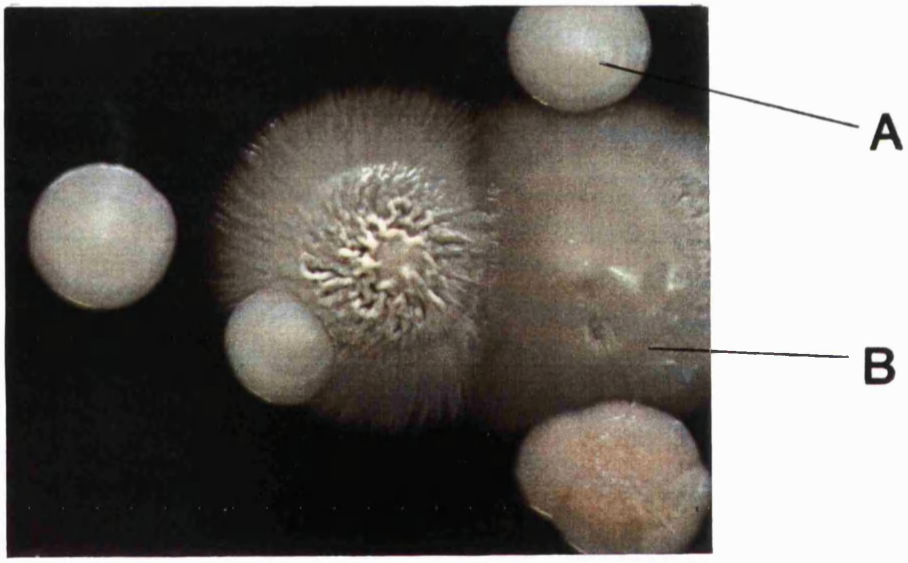
D *Candida tropicals*

E *Candida lipolytica*

F *Rhodotorula glutinis*

G *Sporobolomyces salmonicolor*


C
F
G


A
B

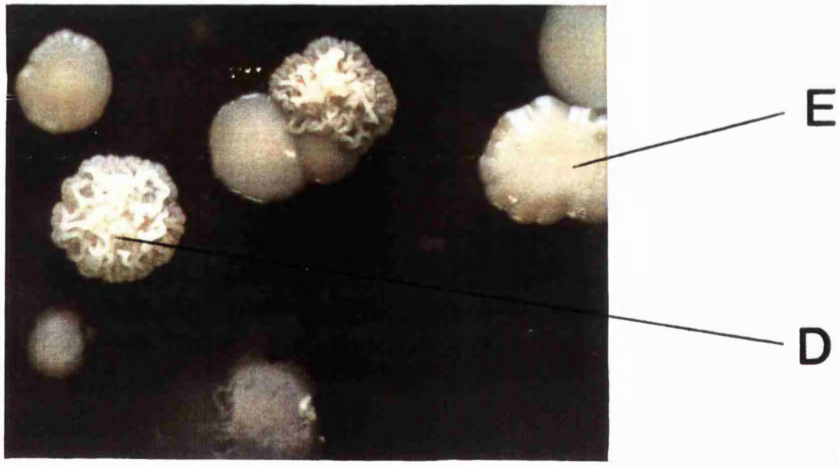

E
D

Figure 4.3 Cell morphology of yeast isolates prepared from wet mounts (x400)

A *Candida japonica*

B *Trichosporon cutaneum*

C *Cryptococcus laurentii*

D *Candida tropicalis*

E *Candida lipolytica*

F *Rhodotorula glutinis*

G *Sporobolomyces salmonicolor*

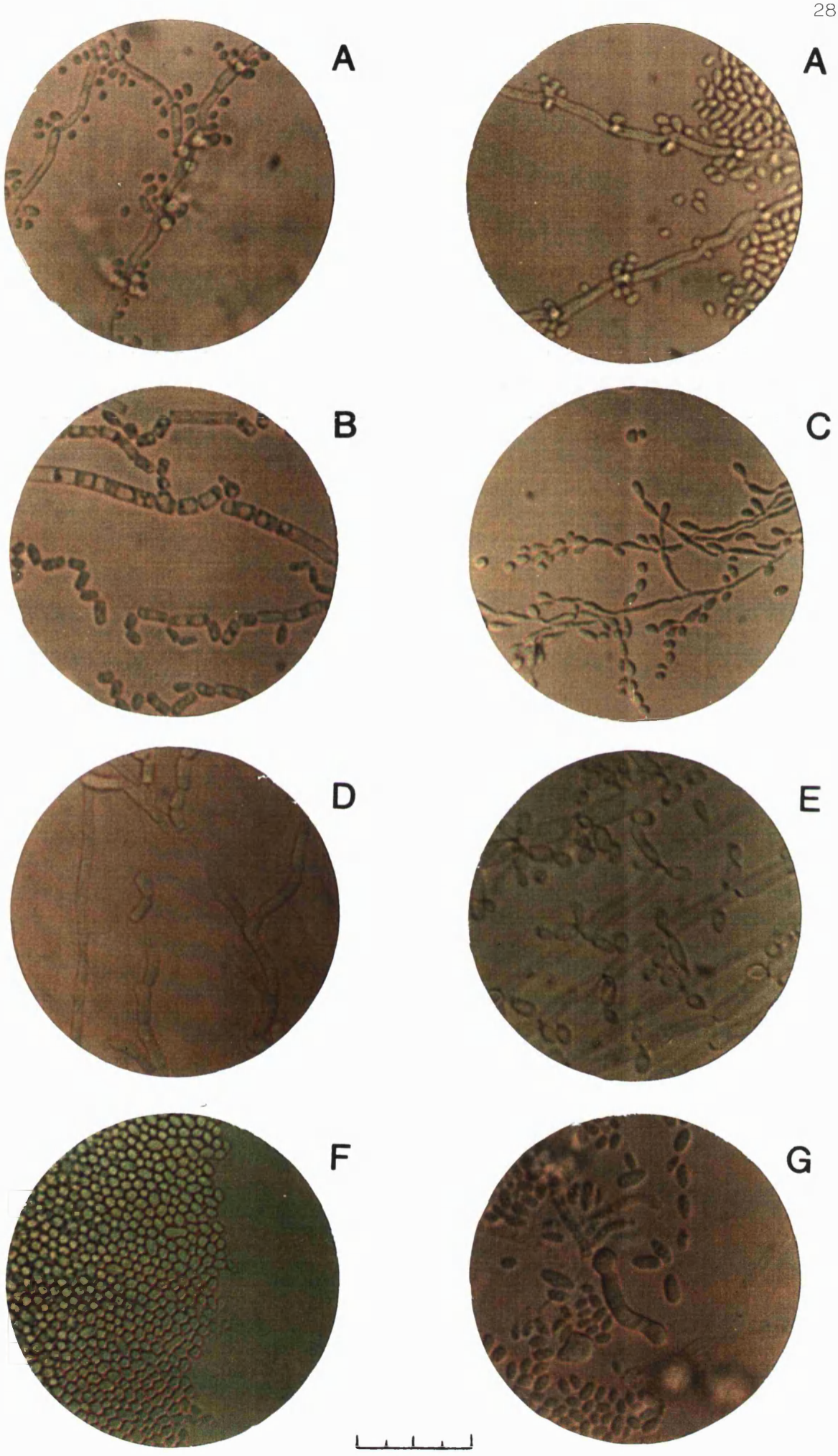

A
A
B
C
D
E
F
G
20µm

Table 4.2 Linear distribution of yeasts in poultry processing plants

Genus Species	Stages of Processing[1] A	B	C	D	E	F
Bullera						
alba	+	+		+		
Candida						
tenuis	+		+		+	
Cryptococcus						
albidus	+	*	+	+	+	+
Debaryomyces						
hansenii	+	+	+	+	+	+
Geotrichum						
candidum	*		+			
Pichia						
burtonii	+	+				
Trichosporon						
aquatile	+					
capitatum	*	+				
cutaneum	*	*	+		+	+
fennicum	+					
figueirae	+					
Candida						
diddensiae		+	+		+	+
Rhodotorula						
glutinis		+	*	*	*	*
minuta		+	+	+		
Candida						
lipolytica			*	*	*	*
Rhodosporidium						
infirmominiatum		+				
Sporidiobolus						
pararoseus					+	
ruinenii		+	+	+		
Sporobolomyces						
holsaticus					+	
salmonicolor			+	+	+	
Candida						
japonica				*	*	*
parapsilosis				+	+	
zeylanoides				+	+	+
Cryptococcus						
laurentii				*	*	*
Tremella						
globospora				+	+	+

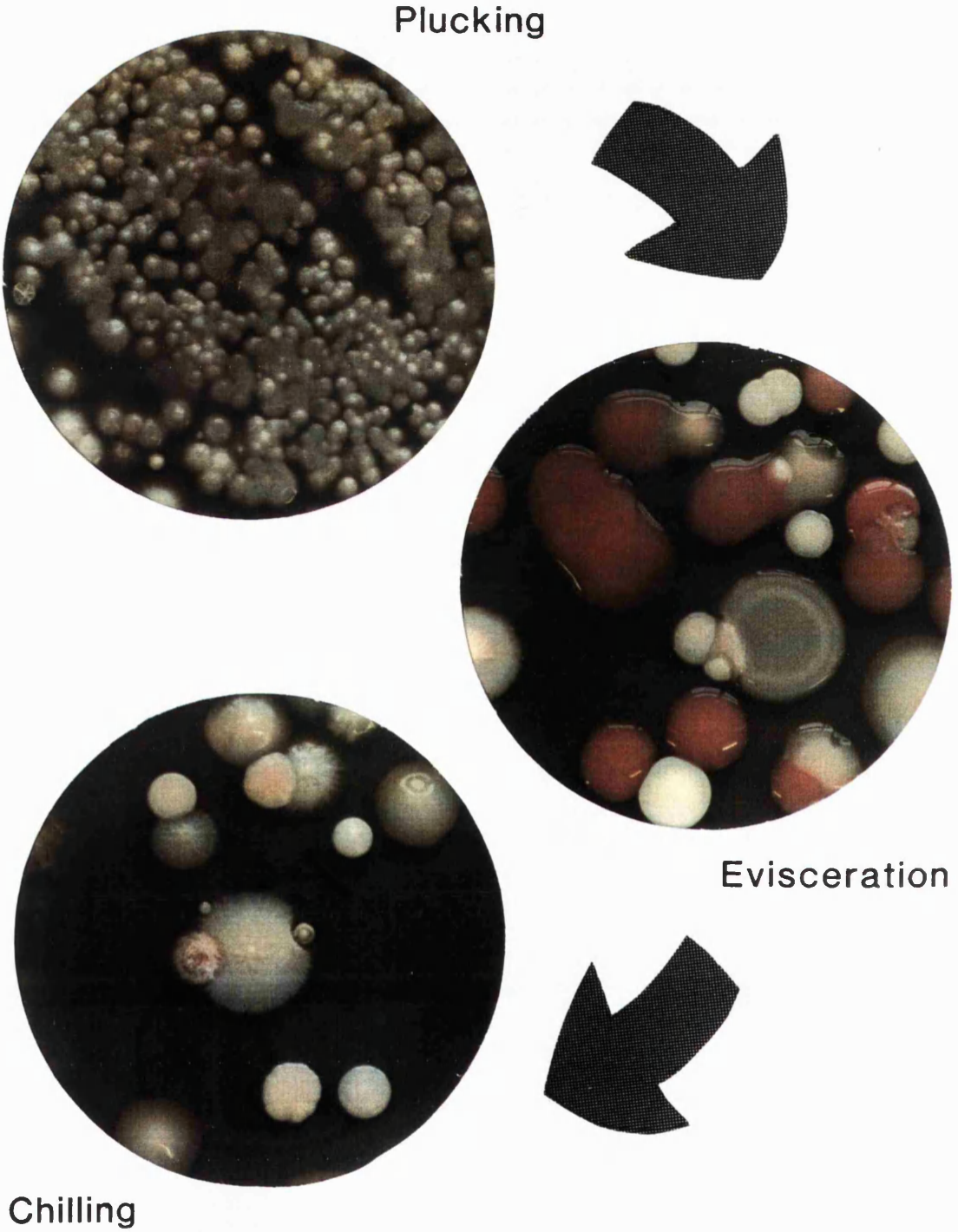
Table 4.2 continued

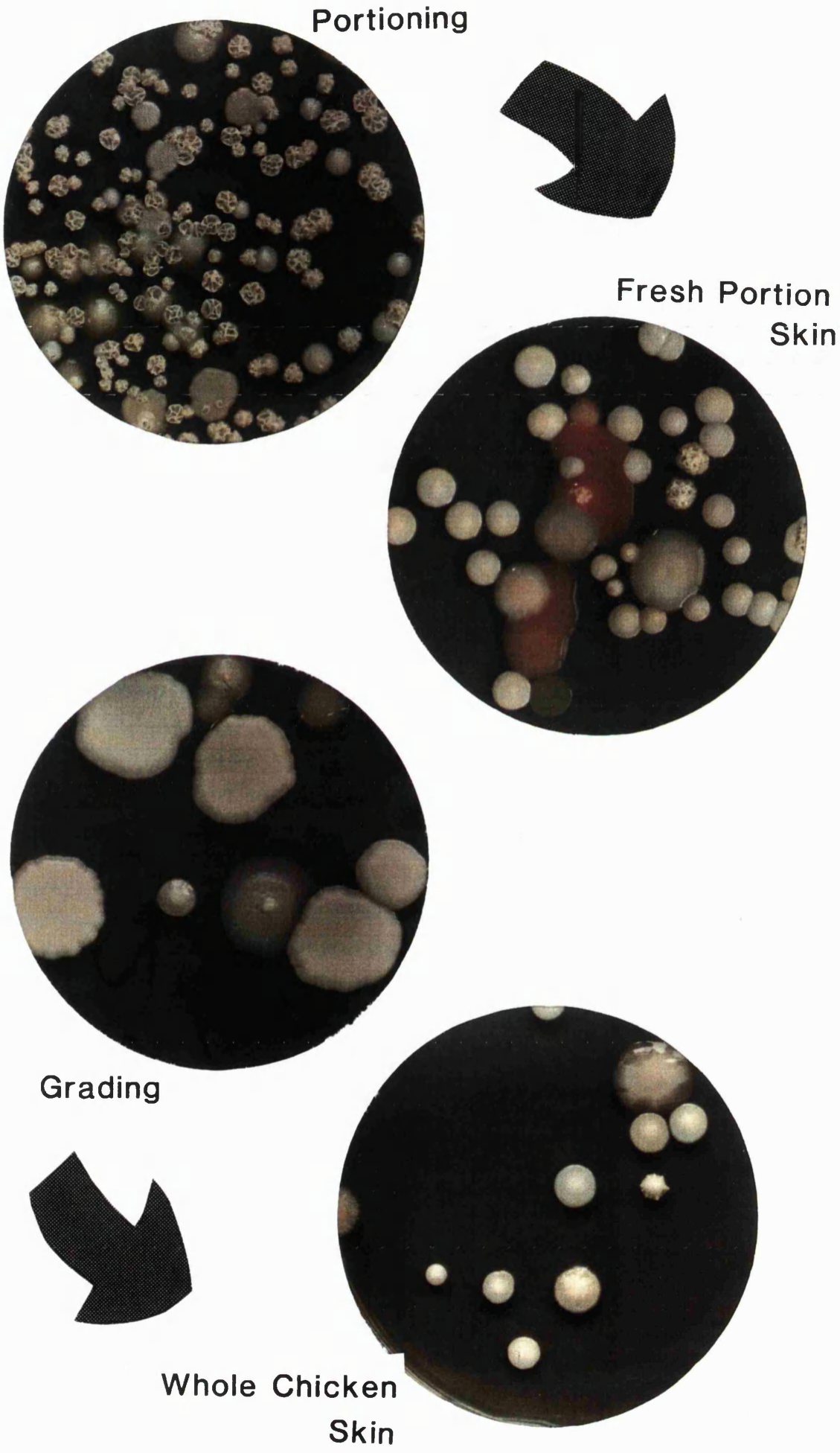
Genus Species	Stages of Processing[1] A	B	C	D	E	F
Candida						
inconspicua					+	
mesenterica					+	
sake					+	
scottii				+	+	
solani					+	
tropicalis					+	
vini					+	
Rhodotorula						
rubra					+	+
Sporobolomyces						
holsaticus					+	

1. A = Entrance bay
   B = Slaughter, bleeding, scalding, plucking
   C = Evisceration, trimming, water-spraying
   D = Chilling
   E = Portioning, freezing, packaging
   F = Weighing, packaging (giblets included) of whole chicken
2. * = Frequently occurring.

Figure 4.4 Linear distribution of yeast on poultry processing equipment

Growth on Oxytetracycline Glucose Yeast Extract Agar after incubation for 5 d at 25°C.




Portioning
Fresh Portion Skin
Grading
Whole Chicken Skin

majority of yeasts isolated from equipment, fresh meat and frozen portions stored for 1 week, were anamorphic, several basidiomycetous teleomorphs were found. These tended to occur on equipment in the chillroom and on meat subjected to extended deep frozen storage.

FACTORY SURVEY

A total of six surveys of two different poultry processing plants sharing a similar layout were made (Figure 4.1). Only the larger one had both an air- and water-chilling facility rather than the traditional water-chiller system. Continuous production occurred such that ca. 50 000 chickens per day were processed; in the larger plant this occurred in 1 x 12 h shifts whereas 3 x 8 h shifts were worked in the smaller plant. The types of yeasts occurring at all stages of processing were determined from swab cultures, water samples and air samples. The range of yeasts could not be quantified. Much of the machinery was moving and therefore difficult to sample by either the template or contact plate methods (McGoldrick et al., 1986).

Trichosporon, Geotrichum, Cryptococcus, Debaryomyces and Candida spp. were isolated (total yeast population, 1.67×10^3 cfu m^{-3}) from air samples taken at the entrance bay where chickens were unloaded before being shackled. A similar range of yeasts was present in air samples taken during slaughter, bleeding and plucking (total yeast population, 8.75×10^2 cfu m^{-3}). At this stage of processing the equipment was contaminated with a range of filamentous and mucoid yeasts (A and B in Table 4.2). Geotrichum

candidum and Trichosporon cutaneum were the most frequently occurring yeasts up to the bleeding stage. The latter organism was commonly the main yeast found during plucking. The rubber fingers of the pluckers were only slightly contaminated whereas the flap doors at the ends of each plucking machine were covered with a white film, even after "cleaning". Trichosporon cutaneum was the only yeast isolated from this film. As every carcass brushed against the flap doors at the entrances and exits of the pluckers, the transfer of this yeast from one carcass to another was assured. At the evisceration stage, the composition of the yeast flora changed significantly. Thus, although T. cutaneum was still isolated, Candida lipolytica and Rhodotorula glutinis became the dominant species on equipment (C in Table 4.2). Additionally, air samples taken from alongside water sprayers showed that this changed yeast flora was in the atmosphere as well as on equipment (4.12×10^2 cfu m^{-3}). These samples also showed a very high mould count (mainly Penicillium and Cladosporium spp.). It is notable that a number of pigmented and/or ballistospore-forming yeasts were isolated at this stage, particularly from the dishes below the evisceration equipment in which spray-water collected. These included, Rhodotorula minuta, Rhodosporidium infirmominiatum, Sporidiobolus ruinenii and Sporobolomyces salmonicolor (C in Table 4.2). All the yeasts noted above tended to be carried over to the chilling process (either chilling water or steel bins in the chilling room). The filamentous yeasts isolated earlier in the processing were rarely detected at this stage. Although C. lipolytica and Rh. glutinis were still commonly found, so too were Cryptococcus

laurentii and Candida japonica (D in Table 4.2). Occasional isolates of other Candida spp. including a cold tolerant yeast, C. scottii, were also obtained. On several occasions an exceptionally slimy yeast, presumptively identified initially with Cryptococcus spp., was isolated from the steel bins in the chill room. This yeast was later identified with Tremella globospora. Air samples contained the yeasts mentioned above, but counts were much lower than in other production areas (1.38×10^2 cfu m^{-3}).

Conveyors and metal slides were swabbed in those areas where chicken portions were prepared and packaged (E in Table 4.2). The dominant yeasts were C. lipolytica, Rh. glutinis, C. japonica and Cr. laurentii. Other species of Candida, especially C. tropicalis which was isolated from the saws used for portioning whole carcasses (see later) and also conveyor belts leaving the blast freezer, occurred sporadically throughout this room. There was a notable absence of the filamentous yeasts common on equipment in earlier stages of processing.

Swabs of metal slides and conveyors where whole chickens were graded, giblets added and carcasses packaged (F in Table 4.2), revealed a yeast flora which differed slightly from that where chickens were portioned. Again, the dominant yeasts were C. lipolytica, Cr. laurentii, C. japonica and Rh. glutinis, but the range of Candida spp. appeared to be curtailed and T. cutaneum occurred more frequently.

In summary, there was a linear distribution of yeast types throughout the processing plant (Figure 4.4). The change in types was most evident at the evisceration stage. Prior to this stage the

yeast flora was predominantly filamentous whereas yeast having smooth colonies became dominant post-evisceration. The latter flora persisted during chilling, along with some cold-tolerant yeasts, and was transferred to subsequent rooms where chickens were packaged. Portioning appeared to introduce a further range of Candida spp. and C. tropicalis was isolated on portioning machinery and conveyors after freezing.

In order to establish any relationship between yeasts on equipment and those on chicken, whole and portioned samples were removed immediately after packaging. General yeast and bacterial counts were determined initially and after chill storage. The types of yeast were identified at this time.

Flora of Fresh Whole Chicken

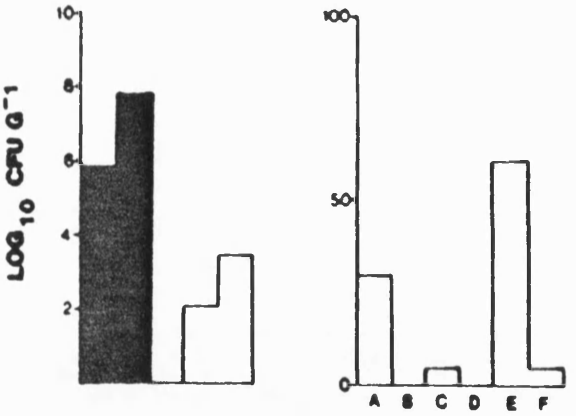
Skin, the visceral cavity, giblets, thigh and breast muscle of fresh whole chicken (3 replicates) were sampled immediately after packaging and after 5 d storage at 6°C. Samples of inner muscle from the thigh and breast did not reveal growth with the detection methods used in this study.

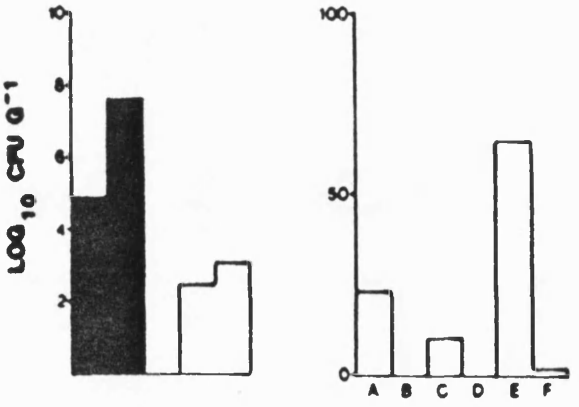
In all cases the initial contamination with bacteria was higher (ca. 1×10^5 cfu g^{-1}) than with yeast (ca. 1×10^2 cfu g^{-1}). Both levels of contamination increased with chill storage so that bacterial counts were ca. 1×10^7 cfu g^{-1} and yeast counts ca. 1×10^3 cfu g^{-1} by 5 days (Figure 4.5). Skin samples were the most highly contaminated initially although, after storage, the counts were similar to those of the visceral cavity and giblet samples. Only four types of yeast were isolated from products and the

Figure 4.5 Contamination of chicken immediately after packaging and after storage.
Yeast flora (%) after storage

WHOLE CHICKEN

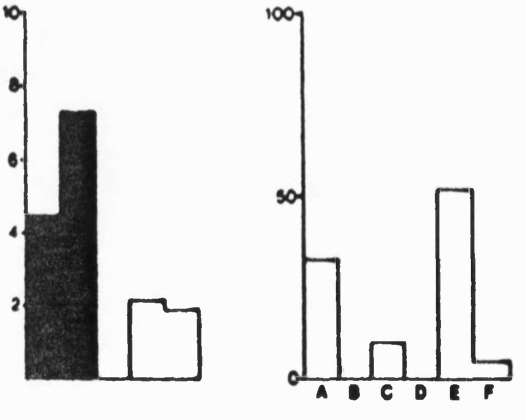
Skin





Visceral Cavity

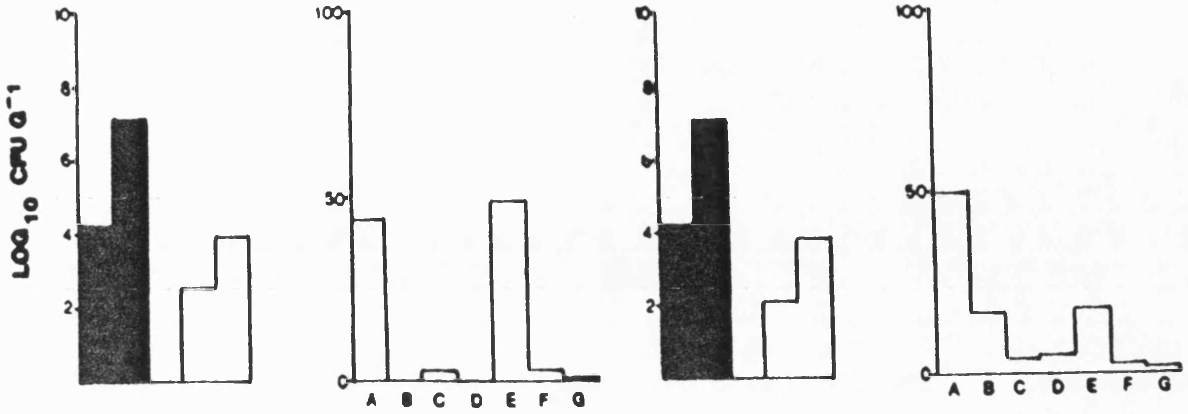
Giblets



FRESH PORTIONS

Skin

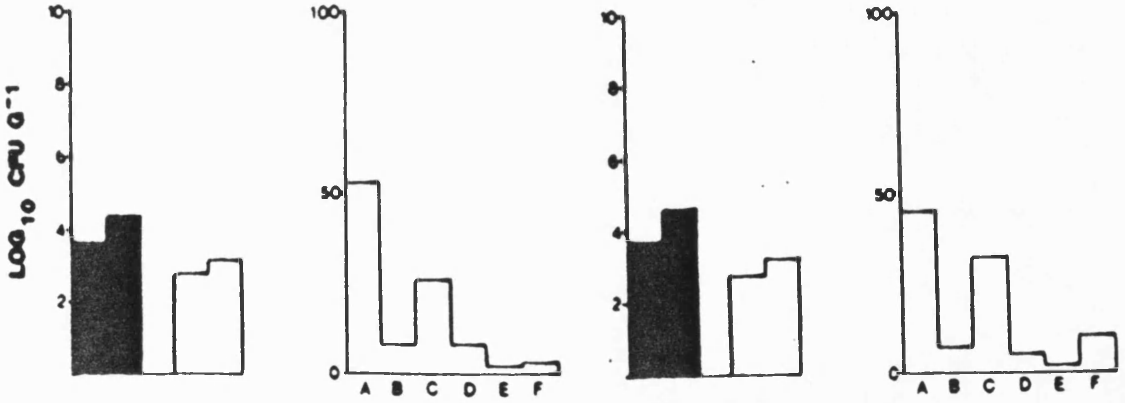
Cut Muscle



FROZEN PORTIONS

Skin

Cut Muscle



■ Bacteria

□ Yeast

A Candida japonica

B Trichosporan cutaneum

C Cryptococcus laurentii

D Candida tropicalis

E Candida lipolytica

F Rhodotorula glutinis

G Sporobolomyces salmonicolor

pattern of occurrence was similar on skin, gut and giblets with (descending order of frequency); Candida lipolytica, Candida japonica, Cryptococcus laurentii and Rhodotorula glutinis. It should be noted that these same four yeasts were also dominant on equipment in the room where whole chickens were graded and packaged (Table 4.2). It may be concluded, therefore, that yeasts from equipment were transferred directly to the carcass during the final stages of poultry processing.

Flora of Fresh Chicken Portions

Skin and muscle from the cut edge of chicken portions (3 replicates) were analysed for general yeast and bacterial counts immediately after packaging and after 5 d storage at 6°C (Figure 4.5). The range and types of yeast were determined after storage.

The initial level of bacterial contamination of portions was slightly lower than that on whole chickens. After storage, yeast contamination of portions was higher. Skin and cut muscle appeared to be contaminated to the same extent (Figure 4.5). The pattern of yeast flora on skin was basically the same as on whole chicken. Candida lipolytica and C. japonica, the dominant yeasts, were present in approximately equal proportions. There were relatively few Cr. laurentii and Rh. glutinis and only a small proportion of Sp. salmonicolor. The pattern on cut muscle was noticeably different to that on skin. Candida japonica was the dominant yeast and the proportions of Candida lipolytica and

Trichosporon cutaneum were similar. Candida tropicalis, a yeast associated with portioning equipment and the conveyor belts of the blast freezer (Table 4.2), Cr. laurentii, Rh. glutinis and Sporobolomyces salmonicolor were also isolated.

The process of portioning chickens led to an increase in the range of contaminating yeasts. Thus, C. tropicalis and Sp. salmonicolor were present on samples from cut muscle but not whole birds. Swabs of the band saw - notoriously difficult to clean - used for portioning, revealed the range of yeasts recovered from the portions but mainly mucoid yeasts together with C. tropicalis and T. cutaneum (E in Table 4.2). Candida lipolytica was the most commonly isolated yeast from whole chicken (Figure 4.5), whereas samples of cut muscle from portions were dominated by Candida japonica and skin samples revealed approximately equal proportions of these two yeasts.

Frozen chicken portions were sampled to determine the effect of freezing on the contaminating flora.

Flora Of Frozen Chicken Portions

Skin and cut muscle of frozen portions were sampled when thawed, immediately after packaging and after storage for 7 d at -18°C. Although the initial bacterial contamination was lower (ca. $1 \times 10^{3.5}$ cfu g^{-1}), levels of yeast contamination were the same as in fresh portions (Figure 4.5). After storage and subsequent thawing, the increase in contamination was less than that of fresh portions. The types of yeast on frozen skin and cut muscle samples were similar to those of fresh cut muscle. It should be noted that

during blast freezing portions were conveyed along a spiral series of belts such that a tumbling motion resulted in considerable contact between the skin and cut muscle of neighbouring portions. Candida japonica was the most frequently isolated yeast. In frozen portions Cryptococcus laurentii was the next most common yeast. This yeast formed a relatively small proportion of the contaminating flora of fresh portions. The remaining yeasts, T. cutaneum, C. tropicalis, C. lipolytica, Rh. glutinis and Sp. salmonicolor, formed only a small proportion of the total yeast flora.

In summary, frozen portions were contaminated with the same types of yeast as fresh portions and thus reflected the contaminating flora of equipment in the portioning and packaging room. In particular, the pattern of yeast growth on both skin and muscle samples of frozen portions resembled that of fresh cut muscle. In the latter, Candida japonica was dominant and the other yeasts were present in relatively small proportions. Freezing appeared to select for Cryptococcus laurentii which was the second most important yeast after C. japonica. Post-processing freezing did not appear to reduce the level of contamination or select against particular yeast types. To determine the effect on yeasts of long-term deep freezing, fresh portions (3 replicates) and neighbouring frozen portions (12 replicates) were removed immediately after processing and stored for periods of up to 3 months.

Effect of Long-Term Frozen Storage on Yeast Flora

General yeast and bacterial counts were obtained (Table 4.3) for the skin and cut muscle of fresh and thawed frozen portions immediately after processing. The remaining portions (9), stored at -18°C, were removed (3) at monthly intervals and thawed overnight before sampling as above (Table 4.3).

Counts were analysed in a 2-factor ANOVA with one factor 'nested' using 'Generalised Linear Modelling' (GLIM). Freezing and site exerted very highly significant ($P < 0.001$) effects on bacterial counts (Table 4.4a). The interaction effect of site and freezing was also highly significant ($P < 0.001$). This indicated a decline in viable bacterial contamination of the skin and cut muscle during extended deep frozen storage (Figure 4.6). A higher proportion of bacteria survived on skin relative to cut muscle, probably because of their location in feather follicles.

Freezing did not exert a significant effect on yeast counts (Table 4.4b) and there was little change in levels of yeast contamination during frozen storage (Figure 4.6). Site exerted a highly significant ($P < 0.001$) effect such that, as with bacteria, samples from skin were more contaminated than those from cut muscle.

The difference in counts for replicate portions sampled at each of the given times (Table 4.3) was highly significant ($P < 0.001$), particularly with yeast counts. The latter effect was a result of the lower yeast (ca. 10^4 cfu g^{-1}) compared with bacterial counts (ca. 10^4 cfu g^{-1}). Although levels of yeast contamination varied between portions, the types of yeast were the same. Indeed, the

Table 4.3 Effect of freezing on yeast and bacterial counts*

	FRESH			FROZEN STORAGE (MTH)											
				0			1			2			3		
Portions	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Yeast															
	2.57	2.40	2.46	2.98	1.90	2.30	2.10	2.13	2.07	2.22	2.89	2.56	2.18	2.41	2.27
Skin	2.76	2.18	2.62	2.93	1.85	2.40	2.22	2.10	2.22	2.42	2.81	2.62	2.16	2.49	2.34
	2.69	2.23	2.56	2.92	1.60	2.41	2.19	2.29	2.20	2.30	2.80	2.41	2.10	2.45	2.42
	2.56	2.32	2.59	2.90	1.85	2.36	2.19	2.35	1.89	2.24	2.88	2.46	2.25	2.37	2.32
		$\bar{x}$ = 2.50			$\bar{x}$ = 2.37			$\bar{x}$ = 2.16			$\bar{x}$ = 2.55			$\bar{x}$ = 2.31	
	2.18	1.00	2.38	3.27	2.11	2.26	1.81	1.56	1.18	1.48	0	1.81	1.75	0	2.18
Cut muscle	2.28	1.30	1.85	3.13	2.15	2.08	1.93	1.32	1.54	1.65	0	1.62	1.65	0	1.93
	2.26	1.00	2.28	3.18	2.04	2.18	1.71	1.18	1.08	1.32	0	1.74	1.93	0	2.82
	2.23	1.69	2.11	3.09	2.00	2.20	1.59	1.45	1.26	1.50	0	1.42	1.89	0	2.00
		$\bar{x}$ = 1.88			$\bar{x}$ = 2.47			$\bar{x}$ = 1.47			$\bar{x}$ = 1.05			$\bar{x}$ = 1.26	
Bacteria															
	4.26	4.34	4.00	3.89	3.73	2.76	2.76	2.65	2.30	1.96	2.31	2.15	1.85	2.25	2.15
Skin	4.11	4.28	4.18	3.73	3.57	3.66	2.22	2.50	2.15	2.02	2.16	2.06	2.16	2.13	2.30
	4.43	4.30	4.26	3.56	3.71	3.51	2.35	2.10	2.26	1.89	2.01	1.98	2.19	1.98	1.96
	4.23	4.34	4.15	3.52	3.66	3.55	2.48	2.12	2.23	2.04	2.22	2.12	2.10	2.06	2.24
		$\bar{x}$ = 4.24			$\bar{x}$ = 3.65			$\bar{x}$ = 2.34			$\bar{x}$ = 2.08			$\bar{x}$ = 2.11	
	4.39	4.05	4.25	3.92	3.57	3.65	2.32	2.06	2.12	1.56	1.96	1.92	0.89	1.11	1.16
Cut muscle	4.28	3.96	4.31	3.86	3.45	3.52	2.45	2.26	2.18	1.82	1.89	1.73	0.76	1.26	1.08
	4.11	4.25	4.35	4.20	3.51	3.46	2.40	2.32	2.25	1.74	1.92	1.96	0.58	0.96	0.81
	4.16	4.10	4.24	3.75	3.30	3.55	2.28	2.28	2.29	1.93	1.71	1.20	1.03	0.91	0.69
		$\bar{x}$ = 4.20			$\bar{x}$ = 3.65			$\bar{x}$ = 2.27			$\bar{x}$ = 1.78			$\bar{x}$ = 0.94	

* Portions (3 replicates) were stored (-18°C) for up to 3 months and thawed before sampling.

Counts = $\log_{10}$ cfu g^{-1} (4 replicates)

$\bar{x}$ = mean values

Table 4.4 Analysis of Variance[+]

(a) Bacteria

Source	df	SS	MS	F	P
Site	1	3.056	3.056	122.84	***
Freeze	4	128.30	32.075	509.61	***
Site x Freeze	4	5.840	1.46	58.68	***
Portion within Freeze	10	0.6294	0.0629	2.53	**
Error	100	2.4879	0.02488		
Total	119	140.33			

(b) Yeast

Source	df	SS	MS	F	P
Site	1	16.96	16,96	125.4	***
Freeze	4	7.966	1.9915	1.46	NS
Site x Freeze	4	8.510	2.1275	15.73	***
Portion within Freeze	10	13.60	1.36	10.057	***
Error	100	13.523	0.13523		
Total	119	60.561			

[+] Statistical analysis = 2 factor ANOVA with 1 factor nested.

Levels of significance: NS = $P > 0.05$, * = $P < 0.05$;

** = $P < 0.01$, *** = $P < 0.001$

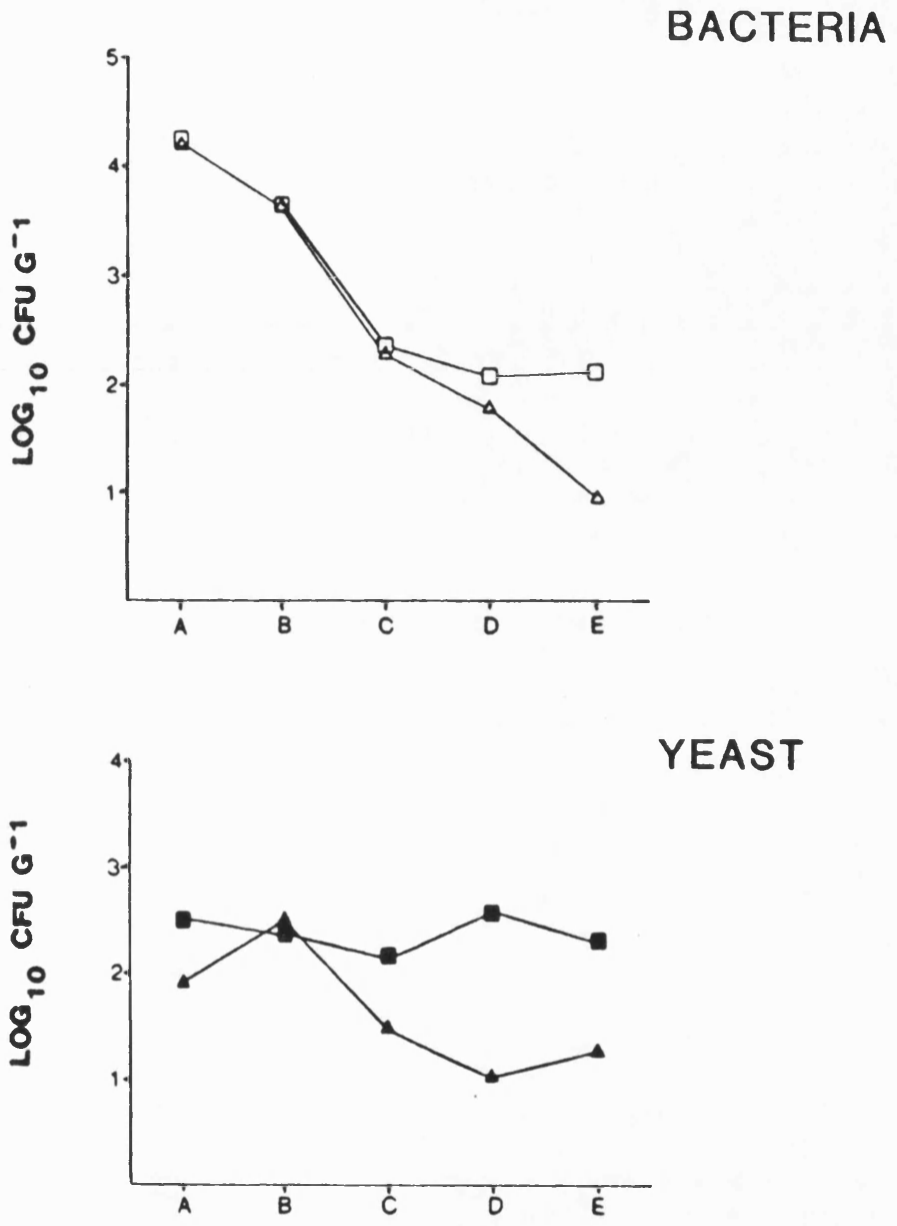


Figure 4.6 Effect of freezing on contamination of chicken portions

Skin: □ Bacteria, ■ Yeast

Cut muscle: △ Bacteria, ▲ Yeast

Storage (mth): A = Fresh (0), B = Frozen (0),

C = Frozen (1), D = Frozen (2),

E = Frozen (3)

yeast flora of portions throughout deep frozen storage was the same as that of portions frozen during processing (Figure 4.5). After 3 months storage (Figure 4.7) Cryptococcus laurentii was the dominant yeast, followed by Candida japonica and Trichosporon cutaneum. The remaining yeasts, Candida tropicalis, Rhodotorula glutinis, Candida lipolytica and Sporobolomyces salmonicolor, were present in relatively small proportions. A small amount of mould growth was also evident.

In conclusion, frozen portions were contaminated with yeasts isolated from equipment used for portioning and packaging. After deep frozen storage (1 wk - 3 mth), it was clearly evident that levels of yeast contamination, particularly on the skin, were little different whereas bacteria rapidly lost viability (Figure 4.6).

Effect of Post-Processing Irradiation on Yeast Flora

Fresh and frozen portions were subjected to a range (0-5 kGy) of γ-irradiation at a dose rate of 10 kGy h^{-1} (Table 4.5). The skin of control and irradiated fresh portions (3 replicates) was sampled after 5 d storage at 6°C whereas frozen portions (3 replicates) were stored for 1 wk at -18°C and thawed overnight before sampling.

Counts were analysed in a 3-way ANOVA using 'Generalised Linear Modelling' (GLIM). Irradiation exerted a highly significant ($P < 0.001$) effect on counts (Table 4.6). As the dose of irradiation was increased there was a corresponding loss in cell viability and no counts were obtained after a dose of 5.00 kGy (Figure 4.8). The effect of irradiation on yeast and bacterial

Table 4.5 Effect of irradiation on the contamination of fresh and frozen chicken portions*

	γ-IRRADIATION (kGy)														
	0			1.25			2.50			3.75			5.00		
Portions	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Fresh															
	2.54	2.81	2.60	1.60	1.00	1.00	1.00	0	0	0	0	0	0	0	0
Yeast	2.49	2.89	2.63	1.60	1.48	1.00	1.00	0	0	0	0	0	0	0	0
	2.52	2.96	2.54	1.70	1.30	1.30	1.30	0	0	0	0	0	0	0	0
	2.38	2.74	2.51	1.70	1.00	1.85	1.00	0	0	0	0	0	0	0	0
		$\bar{x}$ = 2.63			$\bar{x}$ = 1.38			$\bar{x}$ = 0.36			$\bar{x}$ = 0			$\bar{x}$ = 0	
	6.18	6.65	6.19	3.21	3.27	2.08	0	0	1.30	1.00	0	0	0	0	0
Bacteria	6.36	6.84	6.31	3.05	3.45	2.20	0	0	1.00	1.00	0	0	0	0	0
	6.04	6.85	6.16	3.08	3.27	2.15	0	0	1.00	1.00	0	0	0	0	0
	6.20	6.77	6.23	3.15	3.38	2.18	0	0	1.00	1.00	0	0	0	0	0
		$\bar{x}$ = 6.40			$\bar{x}$ = 2.87			$\bar{x}$ = 0.36			$\bar{x}$ = 0.33			$\bar{x}$ = 0	
Frozen															
	2.28	1.60	1.92	1.00	1.00	1.30	1.00	1.00	1.00	1.00	0	0	0	0	0
Yeast	2.34	1.60	1.86	1.00	1.00	1.30	1.00	1.30	1.00	1.00	0	0	0	0	0
	2.48	1.70	1.93	1.00	1.30	1.00	1.00	1.00	1.00	1.00	0	0	0	0	0
	2.41	1.60	2.01	1.00	1.60	1.30	1.00	1.00	1.00	1.03	0	0	0	0	0
		$\bar{x}$ = 1.98			$\bar{x}$ = 1.15			$\bar{x}$ = 1.03			$\bar{x}$ = 0.36			$\bar{x}$ = 0	
	4.57	3.90	3.89	2.04	2.00	1.90	1.00	1.00	1.48	0	0	1.30	0	0	0
Bacteria	4.66	3.90	3.76	2.04	1.60	1.85	1.00	1.00	2.04	0	0	1.48	0	0	0
	4.58	3.70	3.92	1.95	1.95	2.08	1.00	1.00	1.30	0	0	1.00	0	0	0
	4.59	3.78	3.96	1.95	2.15	2.34	1.00	1.00	1.85	0	0	1.00	0	0	0
		$\bar{x}$ = 4.10			$\bar{x}$ = 1.99			$\bar{x}$ = 1.22			$\bar{x}$ = 0.40			$\bar{x}$ = 0	

* Fresh portions were stored at 6°C for 5 d after irradiation; frozen portions were stored at -18°C for 1 week and thawed before the skin was sampled.

Counts = $\log_{10}$ cfu g^{-1} . $\bar{x}$ = mean counts

Table 4.6 Analysis of Variance[+]

Source	df	SS	MS	F	P
Irradiation	3	349.9	116.6	193.2	***
Portions within Irradiation	8	4.828	0.6035	4.59	**
Yeast/Bacteria	1	57.96	57.96	440.7	***
Fresh/Frozen	1	3.347	3.347	25.4	***
Yeast/Bacteria. Fresh/Frozen	1	4.299	4.299	32.69	***
Irradiation. Yeast/Bacteria	3	62.89	20.96	159.4	***
Fresh/Frozen. Irradiation	3	34.12	11.37	86.46	***
Fresh/Frozen. Irradiation. Yeast/Bacteria	3	5.450	1.82	13.84	***
Error	168	22.095	0.1315		
Total	191	544.86			

[+] Statistical analysis = 3 factor ANOVA

Levels of significance: NS = $P > 0.05$, * = $P < 0.05$,

** = $P < 0.01$, *** = $P < 0.001$

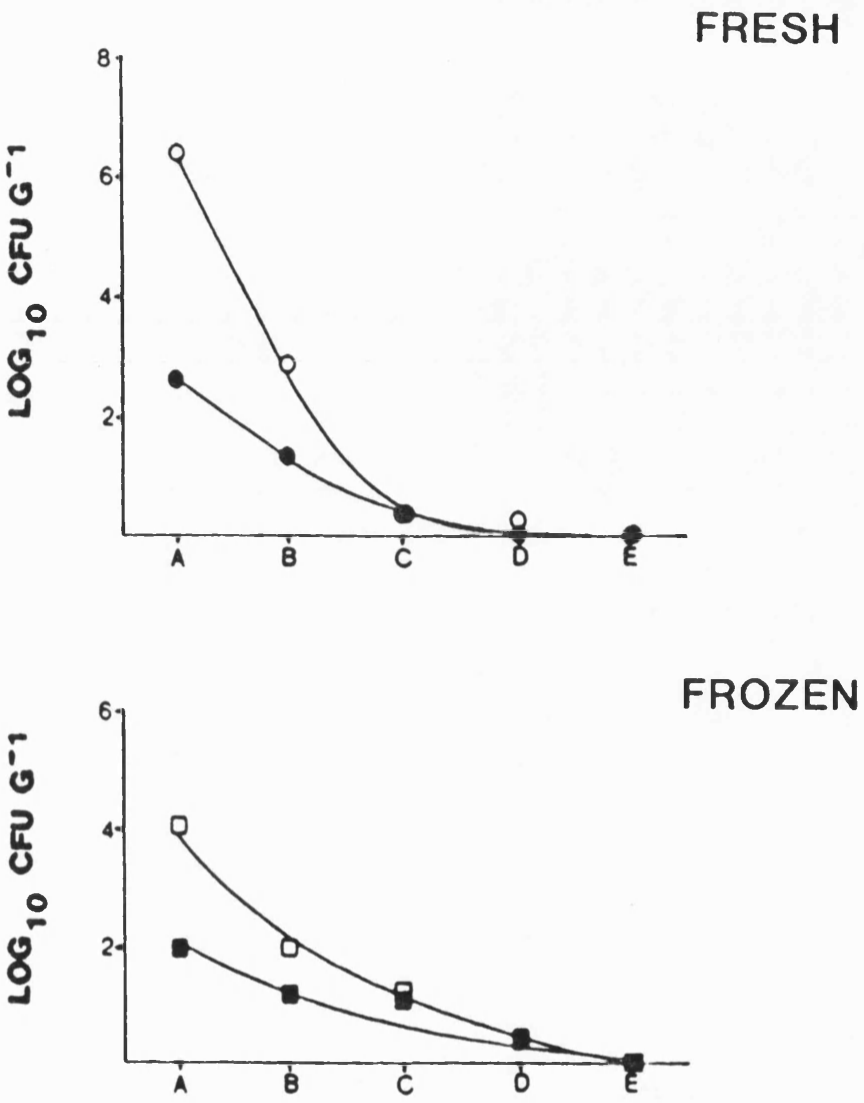


Figure 4.8 Effect of irradiation on contamination of chicken portions

Fresh skin: ○ Bacteria, ● Yeast

Frozen skin: □ Bacteria, ■ Yeast

Irradiation dose (kGy): A = 0, B = 1.25, C = 2.50, D = 3.75, E = 5.00

Mean values of 3 replicates.

counts was very highly significant ($P < 0.001$). Initial levels of bacterial contamination were higher than yeasts in both fresh and frozen portions (Table 4.5). With irradiation doses up to 2.5 kGy, bacteria were killed more rapidly than yeasts (Figure 4.8). The effect of irradiation on contamination of frozen compared with fresh portions was also very highly significant ($P < 0.001$).

In summary, irradiation of fresh and frozen portions resulted in a decrease in contamination. Doses of up to 2.5 kGy appeared to reduce bacterial counts more than those of yeast, particularly with fresh portions (Figure 4.8). It is notable that, in order to reduce the number of contaminating, pathogenic organisms such as Salmonella and Campylobacter spp. on poultry, a pasteurising dose of 2.5 kGy has been recommended. From the present study it may be concluded that with such a dose, the ratio of yeast to bacteria will be increased. After irradiation, storage of fresh or frozen chicken may result in effective competition of yeasts with bacteria and subsequent spoilage.

DISCUSSION

The main objectives of the present study were (1) to determine the types of yeast contaminating equipment in a poultry processing plant, (2) to relate such contamination to the flora of whole and portioned chicken, and (3) to examine the effect of post-processing frozen storage and irradiation on yeast numbers and types.

Two poultry processing plants were investigated and a total of five sampling visits made. The techniques developed for screening and identification described in Chapter 2 permitted the large number of isolates generated from each sampling visit (ca. 100 per visit) to be identified relatively quickly. The plants differed from each other in one important respect, the larger one used both water- and air-chilling rather than solely the more traditional water-chilling method. Even so, analysis revealed the same linear distribution of yeast species contaminating equipment along the processing lines of both plants. Thus, filamentous yeasts were dominant in the early stages of processing whereas a flora comprising predominantly of yeasts with smooth colonies was present following evisceration and during chilling and packaging (Figure 4.1, 4.4).

Filamentous and a smaller proportion of smooth, mainly mucoid yeasts were isolated from surfaces during shackling, slaughter and plucking. Geotrichum candidum and various Trichosporon spp. were commonly found before the feathers of the bird had been soaked (Table 4.2). They were present in the air in both the entrance and

shackling bays. Aerial contamination obviously occurred as birds were taken from crates and shackled. The yeasts noted above are of common occurrence in soils and on animal hides (e.g. Empey and Scott, 1939; Baxter and Illston, 1976; Dalton, 1984; Phaff and Starmer, 1987). It can be concluded that soil yeasts are present in the litter on which broilers are kept and are brought to the processing plant on the feathers of birds (Schefferle, 1965; Romo and Fernandez, 1986).

The range of yeasts decreased and Trichosporon cutaneum and Cryptococcus albidus became the most common isolates once the dead birds had been scalded (51 - 55°C). Whether this change was due to differences in heat and/or chlorine tolerances, or with ability to colonise surfaces was not established. It needs to be stressed that during the initial stages of processing, surfaces are sprayed - sometimes continuously - with chlorinated water. This curtails transfer of contaminants from the slaughter to subsequent processing areas (Fournand et al., 1978). As spraying leads inevitably to extremely wet equipment, microbial colonisation of surfaces exposed to hydraulic flow requires that organisms become firmly attached. In this context, it is notable that Cryptococcus albidus produced copious amounts of extracellular polysaccharide (Figure 4.4) which may conceivably have contributed to adhesion. Trichosporon cutaneum formed a dense white film on rubber flaps at either end of the plucking machine against which every carcass brushed before and after plucking. Even after rigorous cleaning of the plant, these doors remained visually dirty. It is conceivable that yeasts colonised the fissures and cracks of the rubber flaps

and that their filamentous nature permitted outgrowth onto the surface of the flaps after cleaning and during processing.

In retrospect it was unfortunate that gut contents were not included in the surveys because they were probably the source of the different types of yeast which appeared at evisceration. Indeed, the flora of the giblets reflected that on equipment. It is worth noting that the giblets were heavily soiled when the eviscerating equipment ruptured the alimentary canal, a not uncommon event. A range (Table 4.2) of Candida spp. and pigmented yeasts, in particular C. lipolytica and Rh. glutinis, were dominant on equipment at this stage of processing. Although T. cutaneum was still isolated, the incidence of recovery was far lower than at the plucking stage. Dalton (1984), who sampled a pig slaughter line, noted that very few isolates of this yeast were capable of growing at chill temperatures. She concluded that chill temperatures were the principal reason for these organisms failing to colonise equipment used during processing. Abbiss (1978), however, sampled equipment and sausage from a different plant and frequently isolated T. cutaneum throughout the processing areas. Some of the isolates from this study were able to grow at 4°C, but many were not. Thus, tolerance of chill temperatures cannot be the only factor responsible for the reduced incidence of occurrence of T. cutaneum during processing.

The geometry of processing equipment may well play a role in the distribution of yeasts along a processing line. The mechanical eviscerator - a 'two-fingered claw' which pushes down into the visceral cavity and draws out the viscera - harboured Sporidiobolus

ruinenii in places which were difficult to clean. The occurrence of these organisms in dishes beneath the evisceration equipment was attributed to ballistospores being ejected from contaminants in such sites. The same situation probably explains the occurrence of these yeasts in drip water beneath the kidney remover. It is notable that even after cleaning, a small number of these ballistosporous yeasts were recovered from dishes beneath the evisceration equipment. It is possible that during cleaning these organisms survived in niches and crevices and that they subsequently discharged spores which landed on clean equipment.

To reduce the temperature of the carcass from ca. 30°C to 6°C (Mead, 1982), washed eviscerated chickens were either air- or water-chilled. Even under the best conditions of chilling, the inner muscle temperature does not fall sufficiently rapidly to inhibit the growth of psychrotrophic organisms (Rosset, 1982). For this reason, carcasses were sprayed with chlorinated water prior to chilling and the chilling water was also chlorinated (20 ppm). Although yeasts were always isolated from the atmosphere and equipment of the air-chiller, they were isolated on only two occasions from the water-chillers. During the visits when yeasts were not detected, a strong chlorine odour pervaded throughout the processing rooms indicating that exceptionally high concentrations of chlorine had been added to the water. Yeasts isolated from the water-chiller were the same as those obtained from equipment (shackles and sorting bins) in the air-chiller. Additionally, portions which had been water-chilled were contaminated with the same organisms and to the same degree as those which were

air-chilled. This latter observation was in agreement with that of Mead (1975) who examined levels of bacterial contamination of carcasses travelling along the same processing line in which either water- or air-chilling was practised. Extended storage in the air-chiller where meat debris on surfaces has a low a_w might lead to problems since yeasts are better able to survive such conditions than bacteria (Leistner and Rodel, 1976). Thompson et al. (1985) found a high proportion of yeasts on surfaces in the chilling areas of an abattoir, particularly near the cooling systems. There will be a lower a_w around the cooling systems as a result of the higher air velocity at adjacent surfaces (Scott and Vickery, 1939). On one occasion a plant was sampled after cleaning, immediately before processing. The level of contamination in the air-chiller was little different to that recorded in the air-chiller of the plant during the 'cleaning' process. In this case even after effective cleaning, the carcasses would provide a reservoir of organisms which could well be distributed with the movement of air caused by the cooling systems.

Temperature (ca 4°C) and probably a_w were similar in subsequent processing rooms and this may explain why the flora on conveyors and slides in the packaging rooms was similar to that found on equipment in the chilling area. It is notable that the skin of both unfrozen whole and portioned chickens was also contaminated with the same range of yeasts as that found on this equipment, namely; C. japonica, C. lipolytica, Cr. laurentii and Rh. glutinis. The band saws used for portioning whole chickens introduced a further range of yeasts to cut surfaces, in particular, Candida spp. - C.

tropicalis - and Sporobolomyces salmonicolor. These yeasts were never isolated from places where whole carcasses were packaged. In practice, therefore, the band saws may be considered as a peculiar niche in the process because particular yeast types are enriched. Whether this was related to ineffective cleaning during processing, or to conditions favouring the growth of these yeasts during machine use was not established. An example of yeasts colonising niches was described in cider factories (Bowen and Beech, 1967). They noted that although cider apples on the tree were only slightly contaminated with a limited range of yeasts, during harvesting and processing, numbers and types of yeast increased. When 'good manufacturing practices' were achieved, then most of the organisms were eliminated but where standards of hygiene were low, particular yeasts persisted. As these yeasts were found rarely on the fruit and seemed to appear after the milling and pressing stages, it was suggested that they had become established on press cloths and equipment. These organisms were removed after thorough cleaning.

Analyses of the flora of whole and portioned chicken supports the conclusion that contamination was probably a result of transfer of yeasts from equipment to product and vice versa. From the studies on the effects of freezing and irradiation on yeasts, it was apparent that the number of contaminating organisms varied between portions. This occurred even though the portions were obtained at the same time and stage of processing. The fact that only numbers but not types of yeast changed, indicates that portions were coming into contact with equipment on which there was

a heterogenous distribution of contaminants.

As noted previously the skin flora of fresh chicken portions was similar to that of fresh whole chicken. The cut muscle edges of portions, however, harboured a slightly different flora which included a more varied range of Candida spp. and a small proportion of Tr. cutaneum (Table 4.2, Figure 4.5). These yeasts were a feature of both the skin and cut edges of frozen chickens, presumably as a result of attrition between neighbouring portions during the early stages of freezing. It can be concluded therefore that product-to-product transfer of yeasts was also important. Studies investigating adhesion of organisms to chicken skin have shown that organisms may attach irreversibly (Thomas et al., 1987). It is therefore imperative to employ rigorous standards of hygiene such that build-up of contamination on surfaces is avoided (McMeekin and Thomas, 1978; McMeekin et al., 1979).

It was apparent from the investigation of the effects of frozen storage on yeast flora of portions, that short term storage, as may well occur in the home, did not change the types or total numbers of yeast. There was, however, a marked increase in the proportion of Cr. laurentii. This yeast is in fact known to be relatively tolerant of temperatures below zero. Lowry and Gill (1984) noted an increase in the numbers of this organism on lamb stored at -5°C.

Long-term freezing had little effect on levels of yeast contamination whereas there was a rapid loss in viability of bacteria. This was probably due to the drop in a_w which occurs as water is transformed into ice. During commercial freezing ca. 75% of the water becomes ice; however, there is always a certain

proportion of free water remaining, namely 26% at -5°C and 14% at -18°C (Rosset, 1982). According to Leistner and Rödel (1976), at -5°C the a_w is equivalent to 0.990, whereas meat stored at -18°C has an a_w of 0.839. Below -10°C (a_w = 0.907) there are no authentic reports of bacterial growth. Yeasts, however, can tolerate much lower levels of temperature and water activity (Berry and Magoon, 1934; Partmann, 1975). The latter author concludes that most yeasts stop growing at -12 to -15°C but that it is necessary to reach temperatures of -18°C to stop growth completely. One possible example of yeast growth at low temperatures was discussed by McCormack (1951). She recorded 'growth' of a 'pink yeast' in a suitable (non-frozen) medium when oysters were thawed after storage at -34°C ($a_w < 0.75$).

In any population of microbial cells which survive freezing there will still be a range of tolerance of the frozen environment (Tanner and Williamson, 1928; Rosset, 1982). Those of low tolerance will die during storage, this resulting in a gradual selection of the more resistant cells. After an appropriate storage period, only resistant forms will remain and these will form an equilibrium proportion where the change in numbers for a given period becomes progressively less. Once this occurs the microbial population in the meat will remain essentially the same.

Yeasts in the present study tolerated storage at -18°C for three months. There was little change in the total yeast count after this time, whereas bacterial numbers fell considerably. As with short-term storage an increased proportion of the more freeze-tolerant *Cr. laurentii* was evident. It is conceivable that this

yeast would have become the dominant organism with further storage. The number of viable bacterial cells on the skin fell dramatically but appeared to reach a stage of equilibrium after 1 to 2 months. It was probable that survival in feather follicles was responsible for the higher levels of contamination on the skin compared with the exposed cut muscle edge.

According to Rosset (1982), those microorganisms which are resistant to freezing are also resistant to irradiation. From the results discussed above, yeasts might therefore be expected to survive radiation better than bacteria. According to Dawes (1976) there are exceptions, but generally Gram-positive bacteria and yeasts are more resistant than Gram-negative bacteria. As Salmonella are sensitive to irradiation, post-processing treatment of poultry with a pasteurising dose is seen as a possible means of ridding poultry of such pathogenic organisms. A pasteurising dose of 2.5 kGy has been recommended for this purpose since higher levels of irradiation result in a loss of organoleptic quality (Mossel, 1987).

In the present study doses higher than 3.75 kGy on fresh portions resulted in the recovery of a small number of yeasts. Slightly more yeasts and bacteria survived this dose on frozen vis à vis unfrozen portions. James and Werner (1965) concluded that several factors, including freezing, could increase the survival of microbial populations during radiation.

With unfrozen portions it was evident that a dose of 2.5 kGy resulted in a rapid kill-off of bacteria - from ca. 10^6 cfu g^{-1} in control to 10^1 cfu g^{-1} in irradiated portions. Yeasts appeared to

be more resistant, their numbers being reduced from ca.10^3 cfu g^{-1} to 10^1 cfu g^{-1}. It was apparent that a parity in the ratio of yeasts to bacteria occurred after a dose of 2.5 kGy. As discussed in Chapters 1 and 3, equality in the numbers of yeast and bacteria may portend potential spoilage due to yeasts.

When minced beef was radurised with 2.5 kGy, a dose used in the present study, the number of bacterial cells was markedly reduced (Johannsen et al., 1984). These authors found no significant reduction in the overall number of yeast species involved. This was also observed in the present work where Candida japonica was prevalent on fresh portions and C. japonica and Cr. laurentii on frozen portions before and after irradiation. Hughes and Patterson (1988) also found that the microflora of irradiated (2.5 kGy) poultry consisted mainly of yeasts. In view of this last observation it is of particular importance to note that subsequent storage of irradiated minced beef at 4°C resulted in an increase in the number of psychrotrophic yeast cells (Johannsen et al., 1984). In order to determine the potential for yeast spoilage, further studies are required to examine the development of yeast together with bacteria on irradiated (2.5 kGy) chicken stored at chill temperatures.

APPENDIX A - Yeasts in Foods*

Yeast[1]	Types of Food[2]						
	A	B	C	D	E	F	G
Brettanomyces						+	
bruxellensis	+						
intermedius[c]	+		+				
lambicus		+					
naardenevis	+						
Bullera							
alba[c]		+	+			+	
armeniaca		+					
crocea		+					
tsugae						+	
Cyniclomyces							
guttulatus		+					
Candida							
apicola[c]	+	+				+	
apis			+			+	
aquatica						+	
bacarum		+					
blankii			+			+	
boidinii		+					
buffonii						+	
butyri			+				
cacaoi		+					
canterellii[c]	+	+				+	
catenulata[c]	+	+	+			+	
ciferri		+				+	
citrea		+					
curiosa		+				+	
curvata[c]		+	+			+	
dattila[c]	+	+	+			+	+
diddensiae	+					+	
diffluens			+				
diversa[c]		+				+	
ernobii						+	
etchellsii[c]	+	+				+	
famata[c]	+	+	+			+	+
foliorum						+	
fragariorum		+					
friedrichii	+						
fructus		+					
fusiformata		+					
glabrata[c]	+	+	+			+	+
glaebosa			+			+	
globosa[c]	+	+	+			+	+
gropengiesseri						+	
haemulonii						+	

Yeast	Types of Food						
	A	B	C	D	E	F	G
Candida							
hellenica		+					
holmii[c]	+	+				+	+
humicola[c]		+	+	+		+	+
inconspicua[c]	+	+	+			+	+
ingeniosa		+					
ingens		+	+			+	
insectamans						+	
intermedia	+		+		+	+	+
ishiwadae		+					
kefyr[c]		+	+			+	
krusei[c]	+	+	+	+		+	
lactis-condensi	+	+	+				+
lambica[c]			+		+	+	+
lipolytica[c]		+	+	+	+	+	+
magnoliae	+			+			
maltosa		+					
maris							+
melibiosica		+					
melinii		+				+	
membranaefaciens		+					
mesenterica		+	+			+	
molischiana						+	
montana		+					
multis-gemmis		+					
musae		+					
nitrativorans		+					
nodaensis		+					
norvegica						+	
parapsilosis[c]	+	+	+	+	+	+	+
pinus		+	+			+	
pustula		+					
rhagii		+					+
rugosa[c]	+	+	+			+	
sake[c]		+	+	+	+	+	+
salmanticensis							+
silvae						+	
silvatica						+	
silvicultrix						+	
solani						+	
sorbophila		+		+			
sorbosa		+					
sorboxylosa		+					
steatolytica		+				+	
stellata		+	+			+	
tenuis		+				+	

Yeast	Types of Food						
	A	B	C	D	E	F	G
Candida							
tropicalis[c]	+	+	+	+		+	+
utilis[c]	+	+	+				+
valida[c]	+	+	+			+	+
vanderwaltii						+	
versatilis[c]	+	+	+			+	+
vini[c]	+	+	+			+	
wickerhamii						+	
zeylanoides[c]		+	+		+	+	+
Clavispora							
lusitaniae		+	+				
Cryptococcus				+			
albidus[c]		+	+		+	+	+
dimennae						+	+
flavus			+			+	+
gastricus						+	
hungaricus		+				+	+
infirmo-miniatus[c]			+		+	+	+
kuetzingii		+					
laurentii[c]		+	+		+	+	+
luteolus		+	+			+	+
macerans						+	+
neoformans			+			+	+
terreus		+					
Debaryomyces							
castellii						+	
hansenii[c]	+	+	+	+		+	+
marama						+	
polymorphus		+				+	
Filobasidiella							
neoformans		+					
Filobasidium							
capsuligenum[c]		+				+	+
uniguttulatum		+				+	
Geotrichum							
candidum		+	+	+	+		
"fragrans"			+				
Hanseniaspora							
guilliermondii		+					
occidentalis	+	+					
osmophila		+					
uvarum[c]		+				+	
valbyensis		+				+	
vineae		+					

Yeast	Types of Food						
	A	B	C	D	E	F	G
Hansenula							
anomala[c]	+	+	+	+		+	
beijerinckii		+					
beckii							+
californica		+				+	
canadensis		+					
capsulata			+				
holstii		+					
jadinii		+					+
minuta		+					
mrakii		+					
polymorpha						+	
saturnus	+						
silvicola		+					
subpelliculosa[c]	+	+	+				
Issatchenkia							
orientalis[c]	+	+	+	+		+	
terricola		+					
Kloeckera				+			
apiculata[c]	+	+	+			+	
Kluyveromyces							
marxianus[c]	+	+	+				+
thermotolerans[c]	+	+	+			+	+
Leucosporidium							
gelidum							+
scottii[c]		+				+	+
Lodderomyces							
elongisporus	+						
Metschnikowia							
pullcherrima[c]		+				+	
reukaufii[c]	+	+				+	
Nematospora							
coryli		+					
Pichia							
burtonii[c]			+	+		+	+
carsonii[c]						+	
etchellsii[c]		+	+			+	
farinosa[c]		+	+			+	
fermentans[c]	+	+	+		+	+	+
fluxum		+					
guilliermondii[c]	+	+	+			+	
haplophila						+	
heimii		+					

Yeast	Types of Food						
	A	B	C	D	E	F	G
Pichia							
humboldtii		+				+	
kluyveri[c]		+	+				
media						+	
membranaefaciens[c]	+	+	+	+	+	+	+
norvegensis		+	+				
ohmeri[c]	+	+					
onychis[c]		+	+				
pastoris		+					
pijperi[c]		+	+				
rhodanensis		+				+	
strasburgensis							+
toletana			+				
Rhodosporidium							
diobovatum		+					
infirmominiatum[c]		+				+	+
Rhodotorula							
acheniorum		+					
aurantiaca[c]		+					+
glutinis[c]		+	+		+	+	+
graminis		+				+	
lactosa							+
minuta[c]		+	+		+	+	+
rubra[c]	+	+	+	+	+	+	+
Saccharomyces							
"behrensianus"	+						
cerevisiae[c]	+	+	+	+	+	+	+
dairensis		+	+			+	
exiguus[c]	+	+	+		+	+	
kluyveri		+					
servazzii		+					
telluris						+	
Saccharomycodes							
ludwigii		+					
Saccharomycopsis							
capsularis		+					
fibuligera[c]		+		+			
lipolytica[c]			+	+	+	+	+
malanga				+			
Schizosaccharomyces	+						
malidevorans		+					
octosporus		+					
pombe[c]	+	+					

Yeast	Types of Food						
	A	B	C	D	E	F	G
Sporidiobolus							
pararoseus[c]		+				+	
ruinenii						+	
salmonicolor[c]						+	
Sporobolomyces			+	+			
albo-rubescens						+	+
holsaticus		+					
puniceus						+	
roseus[c]	+	+	+			+	
Sterigmatomyces							
nectairii			+				
Torulaspora							
delbrueckii[c]	+	+	+	+	+	+	+
globosa						+	
Trichosporon							
brassicae		+					
capitatum			+			+	
cutaneum[c]		+	+		+	+	+
fennicum		+				+	
fermentans		+				+	
inkin						+	
pullulans[c]		+	+		+	+	+
Wickerhamiella							
domercqii	+	+				+	
Zygosaccharomyces							
bailii[c]	+	+		+	+		
bisporus[c]	+	+					
florentinus	+						
microellipsoides		+					
mrakii		+					
rouxii[c]	+	+	+	+		+	+

1. Yeast species as in Kreger-van Rij (1984).
Geotrichum "fragrans" and *Saccharomyces "behrensianus"* noted from old references (Barnett et al., 1983).

2. Types of Food:
A, sugar-rich ingredients and products; B, fruits and vegetables;
C, milk and dairy products; D, cereal-based products; E, sauces and salads;
F, meat, poultry and other proteinaceous food; G, seafoods.

c Most frequently occurring yeasts.

* Based on Deák and Beuchat (1987) together with all papers quoted in the References.

Appendix B - Yeast Nomenclature

Yeast Nomenclature*

Literature	Text
*Brettanomyces (Br.)**	
sphaericus	*Candida etchellsii*
versatilis	*Candida versatilis*
Candida (C.)	
brumptii	*Candida catenulata*
colliculosa	*Torulaspora delbrueckii*
fennica	*Trichosporon fennicum*
iberica	*Candida zeylanoides*
langeroni	*Candida albicans*
macedonensis	*Candida kefyr*
melibiosi	*Candida guilliermondii*
mycoderma	*Candida vini*
pseudotropicallis	*Candida kefyr*
ravautii	*Candida catenulata*
robusta	*Saccharomyces cerevisiae*
suaveolens	*Candida humicola*
Cryptococcus (Cr.)	
aerius	*Cryptococcus albidus*
curvatus	*Candida curvata*
diffluens	*Cryptococcus albidus*
Debaryomyces (Deb.)	
canterellii	*Debaryomyces polymorphus*
globosus	*Debaryomyces hansenii*
kloeckeri	*Debaryomyces hansenii*
nicotianae	*Debaryomyces hansenii*
subglobosus	*Debaryomyces hansenii*
Endomyces	
fibuliger	*Saccharomycopsis fibuligera*
Endomycopsis (E.)	
bispora	*Hansenula beckii*
burtonii	*Pichia burtonii*
capsularis	*Saccharomycopsis capsularis*
chodati	*Pichia burtonii*
silvicola	*Hansenula silvicola*
Geotrichum	
capitatum	*Trichosporon capitatum*
fermentans	*Trichosporon fermentans*
Hanseniaspora (H'spora)	
melligeri	*Hanseniaspora guilliermondii*
Hansenula (H.)	
angusta	*Hansenula polymorpha*
caprophila	*Hansenula beijerinckii*
schneggii	*Hansenula anomala*

Yeast Nomenclature *

Literature	Text
Hyphopichia	
burtonii	*Pichia burtonii*
Kloeckera (Kl.)	
lafarii	*Kloeckera javanica*
Kluyveromyces (K.)	
bulgaricus	*Kluyveromyces marxianus*
cicerisporus	*Kluyveromyces marxianus*
fragilis	*Kluyveromyces marxianus*
Leucosporidium (Leu.)	
capsuligenum	*Filobasidium capsuligenum*
Moniliella	
suaveolens	*Candida humicola*
Oospora	
lactis	*Geotrichum candidum*
Pichia (P.)	
anomala	*Hansenula anomala*
chodati	*Pichia membranaefaciens*
fibuligera	*Saccharomyces fibuligera*
kudriavzevii	*Issatchenkia orientalis*
nakasei	*Candida citrea*
polymorpha	*Debaryomyces polymorphus*
suaveolens	*Hansenula saturnus*
terricola	*Issatchenkia terricola*
vini	*Pichia carsonii*
Rhodotorula (Rh.)	
ingeniosa	*Candida ingeniosa*
marina	*Rhodotorula minuta*
mucilaginosa	*Rhodotorula rubra*
Saccharomyces (Sacch.)	
acidifaciens	*Zygosaccharomyces bailii*
bailii	*Zygosaccharomyces bailii*
bisporus	*Zygosaccharomyces bisporus*
chevalieri	*Torulaspora delbrueckii*
dairensis	*Zygosaccharomyces rouxii*
delbrueckii	*Torulaspora delbrueckii*
florentinus	*Zygosaccharomyces florentinus*
fragilis	*Kluyveromyces marxianus*
guttulatus	*Cynidomyces guttulatus*
heterogenicus	*Saccharomyces cerevisiae*

Yeast Nomenclature*

Literature	Text
Saccharomyces (Sacch.)	
inusitatus	*Saccharomyces cerevisiae*
italicus	*Saccharomyces cerevisiae*
kloeckerianus	*Torulaspora globosa*
lactis	*Kluyveromyces marxianus*
mellis	*Zygosaccharomyces rouxii*
octosporus	*Schizosaccharomyces octosporus*
oleoginosus	*Saccharomyces cerevisiae*
rosei	*Torulaspora delbrueckii*
rouxii	*Zygosaccharomyces rouxii*
steineri	*Saccharomyces cerevisiae*
torulosus	*Tosulaspora delbrueckii*
tubiformis	*Saccharomyces cerevisiae*
uvarum	*Saccharomyces cerevisiae*
valer	*Torulaspora delbrueckii*
veronae	*Kluyveromyces thermotolerans*
Torula	
cremoris	*Kluyveromyces marxianus*
Torulopsis (T.)	
acris	*Cryptococcus albidus*
aeria	*Cryptococcus albidus*
bovina	*Candida pintolopesii*
candida	*Candida famata*
caroliniana	*Candida lactis-condensi*
cremoris	*Kluyveromyces marxianus*
dattila	*Kluyveromyces thermotolerans*
domercqii	*Candida domercqii*
ernobii	*Candida ernobii*
etchellsii	*Candida etchellsii*
fructus	*Candida fructus*
glabrata	*Candida glabrata*
globosa	*Candida globosa*
gropengiesseri	*Candida gropengiesseri*
haemulonii	*Candida haemulonii*
halonitratophila	*Candida halonitratophila*
holmii	*Candida holmii*
kestonii	*Candida guilliermondii*
lactis-condensi	*Candida lactis-condensi*
mogil	*Zygosaccharomyces rouxii*
molischiana	*Candida molischiana*
nodaensis	*Candida nodaensis*
pseudaeria	*Cryptococcus albidus*
silvatica	*Candida silvatica*
sphaerica	*Kluyveromyces marxianus*
stellata	*Candida stellata*
versatilis	*Candida versatilis*
wickerhamii	*Candida wickerhamii*

Yeast Nomenclature [+]

Literature	Text
Trichosporon (Tr.)	
beigelii	*Trichosporon cutaneum*
penicillatum	*Geotrichum penicillatum*
variabile	*Pichia burtonii*
Willopsis	
beijerinckii	*Hansenula beijerinckii*
californica	*Hansenula californica*
mrakii	*Hansenula mrakii*
saturnus	*Hansenula saturnus*
Yarrowia	
lipolytica	*Saccharomyces lipolytica*
Zygosaccharomyces (Zygosacch.)	
barkeri	*Zygosaccharomyces rouxii*
globiformis	*Torulaspora delbrueckii*
halomembranis	*Zygosaccharomyces rouxii*
japonicus	*Zygosaccharomyces rouxii*
major	*Zygosaccharomyces rouxii*
nussbaumeri	*Zygosaccharomyces rouxii*
pastoris	*Pichia pastoris*
rugosus	*Zygosaccharomyces rouxii*
salsus	*Zygosaccharomyces rouxii*
soya	*Zygosaccharomyces rouxii*
variabilis	*Zygosaccharomyces rouxii*

Sexual/Perfect [°]	Asexual/Imperfect
Citeromyces matritensis	*Candida globosa*
Clavispora lusitataniae	*Candida lusitaniae*
Debaryomyces hansenii	*Candida famata*
Dekkera bruxellensis	*Brettanomyces bruxellensis*
intermedia	*intermedia*
Filobasidiella neoformans	*Cryptococcus neoformans*
Filobasidium capsuligenum	*Candida japonica*
uniguttulatus	*Cryptococcus unigultulatus*
Hanseniaspora guilliermondii	*Kloeckera apis*
occidentalis	*javanica*
osmophila	*corticis*
uvarum	*apiculata*

Sexual/Perfect°	Asexual/Imperfect
Hanseniaspora guilliermondii	*Kloeckera apis*
valbyensis	*japonica*
vineae	*africana*
Hansenula anomala	*Candida pelliculosa*
canadensis	*melinii*
capsulata	*molischiana*
holstii	*silvicola*
jadinii	*utilis*
Issatchenkia occidentalis	*Candida sorbosa*
orientalis	*krusei*
Kluyveromyces marxianus	
var. marxianus	*Candida kefyr*
var. lactis	*sphaerica*
Kluyveromyces thermotolerans	*Candida dattila*
Leucosporidium gelidum	*Candida gelida*
scottii	*scottii*
Metschnikowia reukaufii	*Candida reukaufii*
Pichia burtonii	*Candida variabilis*
fermentans	*lambica*
guilliermondii	*guilliermondii*
humboldtii	*ingens*
membranaefaciens	*valida*
norvegensis	*norvegensis*
Rhododporidium capitatum	*Cryptococcus infirmo-miniatus*
diobovatum	*Rhodotorula glutinis*
infirmominiatum	*Cryptococcus infirmo-miniatus*
Saccharomyces exiguus	*Candida holmii*
telluris	*pintolopesii*
Saccharomyces lipolytica	*Candida lipolytica*
Sarcinosporon inkin	*Trichosporon inkin*
Sporidiobolus pararoseus	*Sporobolomyces shibatanus*
salmonicolor	*salmonicolor*
Wickerhamiella domercqii	*Candida domercqii*

+ Recent and/or accepted names found in Kreger-van Rij (1984) and used in the text.

(a) Generic abbreviations used in the text.

° Yeasts listed below are represented in two genera according to whether or not they are known to reproduce sexually.

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